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(54) Title: DNA REPLICATION PROTEINS OF GRAM POSITIVE BACTERIA AND THEIR USE TO SCREEN FOR CHEMICAL INHIBITORS

(57) Abstract: The present invention relates to alpha-large, alpha-small, delta, delta prime, tau, beta, SSB, DnaG DnaB encoding genes from Gram positive bacterium, preferably *Streptococcus* and *Staphylococcus* bacterium. The formation of functional polymerase as well as the use of such a polymerase in sequencing and amplification is also disclosed. The individual genes and proteins or polypeptides are useful in identification of compounds with antibiotic activity.

DNA REPLICATION PROTEINS OF GRAM POSITIVE BACTERIA AND THEIR USE TO SCREEN FOR CHEMICAL INHIBITORS

The present application is a continuation-in-part of U.S. Patent Application Serial No. 09/235,245 filed January 22, 1999, which claims benefit of U.S. Provisional Patent Application Serial No. 60/093,727 filed July 22, 1998, and U.S. Provisional Patent Application Serial No. 60/074,522 filed January 22, 1998, all of which are hereby incorporated by reference. The present application also claims benefit of U.S. Provisional Patent Application Serial No. 60/146,178 filed July 29, 1999, which is hereby incorporated by reference.

The present invention was made with funding from National Institutes of Health Grant No. GM38839. The United States Government may have certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to genes and proteins that replicate the chromosome of Gram positive bacteria. These proteins can be used in sequencing, amplification of DNA, and in drug discovery to screen large libraries of chemicals for identification of compounds with antibiotic activity.

BACKGROUND OF THE INVENTION

All forms of life must duplicate the genetic material to propagate the species. The process by which the DNA in a chromosome is duplicated is called replication. The replication process is performed by numerous proteins that coordinate their actions to duplicate the DNA smoothly. The main protein actors are as follows (reviewed in Kornberg et al., DNA Replication, Second Edition, New York: W.H. Freeman and Company, pp. 165-194 (1992)). A helicase uses the energy of ATP hydrolysis to unwind the two DNA strands of the double helix. Two copies of the DNA polymerase use each "daughter" strand as a template to convert them into two new duplexes. The DNA polymerase acts by polymerizing the four monomer unit building blocks of DNA (the 4 dNTPs, or deoxynucleoside triphosphates are: dATP, dCTP, dGTP, dTTP). The polymerase rides along one strand of DNA using it as a

template that dictates the sequence in which the monomer blocks are to be polymerized. Sometimes the DNA polymerase makes a mistake and includes an incorrect nucleotide (e.g., A instead of G). A proofreading exonuclease examines the polymer as it is made and excises building blocks that have been improperly inserted in the polymer.

Duplex DNA is composed of two strands that are oriented antiparallel to one another, one being oriented 3'-5' and the other 5' to 3'. As the helicase unwinds the duplex, the DNA polymerase moves continuously forward with the helicase on one strand (called the leading strand). However, due to the fact that DNA polymerases can only extend the DNA forward from a 3' terminus, the polymerase on the other strand extends DNA in the opposite direction of DNA unwinding (called the lagging strand). This necessitates a discontinuous ratcheting motion on the lagging strand in which the DNA is made as a series of Okazaki fragments. DNA polymerases cannot initiate DNA synthesis *de novo*, but require a primed site (i.e., a short duplex region). This job is fulfilled by primase, a specialized RNA polymerase, that synthesizes short RNA primers on the lagging strand. The primed sites are extended by DNA polymerase. A single-stranded DNA binding protein ("SSB") is also needed; it operates on the lagging strand. The function of SSB is to coat single stranded DNA ("ssDNA"), thereby melting short hairpin duplexes that would otherwise impede DNA synthesis by DNA polymerase.

The replication process is best understood for the Gram negative bacterium *Escherichia coli* and its bacteriophages T4 and T7 (reviewed in Kelman et al., "DNA Polymerase III Holoenzyme: Structure and Function of Chromosomal Replicating Machine," Annu. Rev. Biochem., 64:171-200 (1995); Marians, K.J., "Prokaryotic DNA Replication," Annu. Rev. Biochem., 61:673-719 (1992); McHenry, C.S., "DNA Polymerase III Holoenzyme: Components, Structure, and Mechanism of a True Replicative Complex," J. Bio. Chem., 266:19127-19130 (1991); Young et al., "Structure and Function of the Bacteriophage T4 DNA Polymerase Holoenzyme," Am. Chem. Soc., 31:8675-8690 (1992)). The eukaryotic systems of yeast (*Saccharomyces cerevisae*) (Morrison et al., "A Third Essential DNA Polymerase in *S. cerevisiae*," Cell, 62:1143-51 (1990) and humans (Bambara et al., "Reconstitution of Mammalian DNA Replication," Prog. Nuc. Acid Res.," 51:93-123 (1995)) have also been characterized in some detail as has herpes virus (Boehmer et al., "Herpes

Simplex Virus DNA Replication," Annu. Rev. Biochem., 66:347-384 (1997)) and vaccinia virus (McDonald et al., "Characterization of a Processive Form of the Vaccinia Virus DNA Polymerase," Virology, 234:168-175 (1997)). The helicase of *E. coli* is encoded by the *dnaB* gene and is called the DnaB-helicase. In phage T4, the helicase is the product of the gene 41, and, in T7, it is the product of gene 4. Generally, the helicase contacts the DNA polymerase in *E. coli*. This contact is necessary for the helicase to achieve the catalytic efficiency needed to replicate a chromosome (Kim et al., "Coupling of a Replicative Polymerase and Helicase: A tau-DnaB Interaction Mediates Rapid Replication Fork Movement," Cell, 84:643-650 (1996)). The identity of the helicase that acts at the replication fork in a eukaryotic cellular system is still not firm.

The primase of *E. coli* (product of the *dnaG* gene), phage T4 (product of gene 61), and T7 (gene 4) require the presence of their cognate helicase for activity. The primase of eukaryotes, called DNA polymerase alpha, looks and behaves differently. DNA polymerase alpha is composed of 4 subunits. The primase activity is associated with the two smaller subunits, and the largest subunit is the DNA polymerase which extends the product of the priming subunits. DNA polymerase alpha does not need a helicase for priming activity on single strand DNA that is not coated with binding protein.

The chromosomal replicating DNA polymerase of all these systems, prokaryotic and eukaryotic, share the feature that they are processive, meaning they remain continuously associated with the DNA template as they link monomer units (dNTPs) together. This catalytic efficiency can be manifest *in vitro* by their ability to extend a single primer around a circular ssDNA of over 5,000 nucleotide units in length. Chromosomal DNA polymerases will be referred to here as replicases to distinguish them from DNA polymerases that function in other DNA metabolic processes and are far less processive.

There are three types of replicases known thus far that differ in how they achieve processivity and how their subunits are organized. These will be referred to here as Types I-III. The Type I is exemplified by the phage T5 replicase, which is composed of only one subunit yet is highly processive (Das et al., "Mechanism of Primer-template Dependent Conversion of dNTP-dNMP by T7 DNA Polymerase," J. Biol. Chem., 255:7149-7154 (1980)). It is possible that the T5 enzyme achieves

processivity by having a cavity within it for binding DNA, with a domain of the protein acting as a lid that opens to accept the DNA and closes to trap the DNA inside, thereby keeping the polymerase on DNA during polymerization of dNTPs. Type II is exemplified by the replicases of phage T7, herpes simplex virus, and vaccinia virus. In these systems, the replicase is composed of two subunits, the DNA polymerase and an "accessory protein" which is needed for the polymerase to become highly efficient. It is presumed that the DNA polymerase binds the DNA in a groove and that the accessory protein forms a cap over the groove, trapping the DNA inside for processive action. Type III is exemplified by the replicases of *E. coli*, phage T4, yeast, and humans in which there are three separate components, a sliding clamp protein, a clamp loader protein complex, and the DNA polymerase. In these systems, the sliding clamp protein is an oligomer in the shape of a ring. The clamp loader is a multiprotein complex which uses ATP to assemble the clamp around DNA. The DNA polymerase then binds the clamp which tethers the polymerase to DNA for high processivity. The replicase of the *E. coli* system contains a fourth component called tau that acts as a glue to hold two polymerases and one clamp loader together into one structure called Pol III*. In this application, any replicase that uses a minimum of three components (i.e., clamp, clamp loader, and DNA polymerase) will be referred to as either a three component polymerase, a type III enzyme, or a DNA polymerase III-type replicase.

The *E. coli* replicase is also called DNA polymerase III holoenzyme. The holoenzyme is a single multiprotein particle that contains all the components; it is comprised of ten different proteins. This holoenzyme is suborganized into four functional components called: 1) Pol III core (DNA polymerase); 2) gamma complex or tau/gamma complex (clamp loader); 3) beta subunit (sliding clamp); and 4) tau (glue protein). The DNA polymerase III "core" is a tightly associated complex containing one each of the following three subunits: 1) the alpha subunit is the actual DNA polymerase (129 kDa); 2) the epsilon subunit (28 kDa) contains the proofreading 3'-5' exonuclease activity; and 3) the theta subunit has an unknown function. The gamma complex is the clamp loader and contains the following subunits: gamma, delta, delta prime, chi and psi (U.S. Patent No. 5,583,026 to O'Donnell). Tau can substitute for gamma, as can a tau/gamma heterooligomer. The beta subunit is a homodimer and forms the ring shaped sliding clamp. These

components associate to form the holoenzyme and the entire holoenzyme can be assembled *in vitro* from 10 isolated pure subunits (U.S. Patent No. 5,583,026 to O'Donnell; U.S. Patent No. 5,668,004 to O'Donnell). The *E. coli dnaX* gene encodes both tau and gamma. Tau is the product of the full gene. Gamma is the product of the first 2/3 of the gene; it is truncated by an efficient translational frameshift that results in incorporation of one unique residue followed by a stop codon.

The tau subunit, encoded by the same gene that encodes gamma (*dnaX*), also acts as a glue to hold two cores together with one gamma complex. This subassembly is called DNA polymerase III star (Pol III*). One beta ring interacts with each core in Pol III* to form DNA polymerase III holoenzyme.

During replication, the two cores in the holoenzyme act coordinately to synthesize both strands of DNA in a duplex chromosome. At the replication fork, DNA polymerase III holoenzyme physically interacts with the DnaB helicase through the tau subunit to form a yet larger protein complex termed the "replisome" (Kim et al., "Coupling of a Replicative Polymerase and Helicase: A tau-DnaB Interaction Mediates Rapid Replication Fork Movement," Cell, 84:643-650 (1996); Yuzhakov et al., "Replisome Assembly Reveals the Basis for Asymmetric Function in Leading and Lagging Strand Replication," Cell, 86:877-886 (1996)). The primase repeatedly contacts the helicase during replication fork movement to synthesize RNA primers on the lagging strand (Marians, K.J., "Prokaryotic DNA Replication," Annu. Rev. Biochem., 61:673-719 (1992)).

Intensive subtyping of prokaryotic cells has now lead to a taxonomic classification of prokaryotic cells as eubacteria (true bacteria) to distinguish them from archaebacteria. Within eubacteria are many different subcategories of cells, although they can broadly be subdivided into Gram positive - and Gram negative-like cells. Numerous complete and partial genome sequences of prokaryotes have appeared in the public databases.

In the present invention, new genes from the Gram positive bacteria, *Streptococcus pyogenes* (e.g., *S. pyogenes*) and *Staphylococcus aureus* (e.g., *S. aureus*) are identified. They are assigned names based on their nearest homology to subunits in the *E. coli* system. The genes encoding *E. coli* replication proteins are as follows: alpha (*dnaE*); epsilon (*dnaQ*); theta (*holE*); tau (full length *dnaX*); gamma

(frameshift product of *dnaX*); delta (*holA*); delta prime (*holB*); chi (*holC*); psi (*holD*); beta (*dnaN*); DnaB helicase (*dnaB*); and primase (*dnaG*).

Study of the organisms for which a complete genome sequence is available reveals that no organism has identifiable homologues to all the subunits of the *E. coli* three component polymerase, Pol III holoenzyme (see Table 1 below). All other organisms lack the θ subunit (*holE*), and all except one lack genes encoding the χ and ψ subunits (*holC* and *holD*, respectively) as judged by sequence comparison searches. Further, the α and ε subunits are fused into one large α subunit in some organisms (e.g., Gram positive cells) as detailed in (Sanjanwala et al., "DNA Polymerase III Gene of *Bacillus subtilis*," Proc. Natl. Acad. Sci., USA, 86:4421-4424 (1989)). Although all organisms have homologues to τ, β, δ' and SSB, the δ subunit has diverged significantly (either not recognized or nearly not recognized by gene searching programs), perhaps even to the point where it is no longer involved in DNA replication. The DnaX product also would appear to lack frameshift signals in most organisms. This predicts only one protein (tau) will be produced from this gene, instead of two as in *E. coli*. Indeed, this has been shown to be true for the *Staphylococcus aureus* DnaX (U.S. Patent Application Serial No. 09/235,245, which is hereby incorporated by reference). Finally, genetic study of *Bacillus subtilis* identified two genes that do not have counterparts in *E. coli* (*dnaB*, not the helicase, and *dnaH*) as well as one other gene, *dnaI*, that is only very distantly related to *E. coli dnaC* (Karamata et al., "Isolation and Genetic Analysis of Temperature-Sensitive Mutants of *B. subtilis* Defense in DNA Synthesis," Molec. Gen. Genet., 108:277-287 (1970); Braund et al., "Nucleotide Sequence of the *Bacillus subtilis dnaD* Gene," Microb., 141:321-322 (1995); Hoshino et al., "Nucleotide Sequence of *Bacillus subtilis dnaB*: A Gene Essential for DNA Replication Initiation and Membrance Attachment," Proc. Natl. Acad. Sci. USA," 84:653-657 (1987)). Keeping in mind the apparently random, or at least unpredictable process of evolution, it is possible that these apparently new genes perform novel functions that may result in a new type of polymerase for chromosomal replication. Thus, it seems possible that new proteins may have evolved to take the place of χ, ψ, θ, the frameshift product of DnaX, and possibly δ in other eubacteria. These considerations indicate that the three component polymerase of different eubacteria may have different structures. That this may be so would not be surprising as different bacteria are often less related evolutionarily than plants are to

humans. For example, the split between Gram positive and Gram negative bacteria occurred about 1.2 billion years ago. This distant split makes Gram positive cells an attractive source to examine how different other eubacterial three component polymerases are from the *E. coli* Pol III holoenzyme.

Table 1

Organism (Order)	χ	ψ	θ	ε	α	β	dnaX	δ'	δ	
Escherichia coli Proteobacteria	+	+	+	+	+	+	+	+	+	
Haemophilus influenzae Proteobacteria	+	+	_	+	+	+	+	+	+	
Mycoplasma genitalium Firmicutes	_	_	_	_	+	+	+	+	+	(weak)
Synichisystis sp. Cyanobacteria	_	_	_	_	+	+	+	+	+	(weak)
Bacillus subtilis Firmicutes	_	_	_	_	+	+	+	+	+	(weak)
Borrelia burgdorferi Spirochaetales	_	_	_	_	+	+	+	+	+	(weak)
Aquifex aeolicus Aquificales	_	_	_	+	+	+	+	+	+	(weak)
Mycobacterium tuberculosis Firmicutes & Actinobacteria	_	_	_	+	+	+	+	+	+	(weak)
Treponema pallidum Spirochaetales	_	_	_	+	+	+	+	+	+	(weak)
Chlamydia trachomatis Chlamydiales	_	_	_	+	+	+	+	+	+	(weak)
Rickettsia prowazekii Proteobacteria	_	_	_	+	+	+	+	+	+	(weak)
Helicobacter pylori Proteobacteria	_	_	_	+	+	+	+	+	+	(weak)
Thermatoga maritima Thermotogales	_	_	_	_	+	+	+	+	+	(weak)

The goal of this invention is to learn how to form a functional three component polymerase from an organism that is highly divergent from *E. coli* and whether it is as rapid and processive as the *E. coli* Pol III holoenzyme. Namely, from bacteria lacking χ, ψ, or θ, or having a widely divergent δ subunit, or having only one DnaX product, or an α subunit that encompasses both α and ε activities. All eubacteria for which the entire genome has been sequenced have at least one of these differences from *E. coli*. Many Gram negative bacteria have one or more of these differences (e.g., *Haemophilus influenzae* and *Aquifex aeolicus*). Bacteria of the Gram positive class have all of these different features. Because of the distant

evolutionary split between Gram positive and Gram negative bacteria, their mechanisms of replication may have diverged significantly as well. Indeed, purification of the replication polymerase from *B. subtilis*, a Gram positive cell, gives only a single subunit polymerase (Barnes et al., "Purification of DNA Polymerase III of Gram-Positive Bacteria," Methods Enzy. 262:35-42 (1995); Barnes et al., "Antibody to B. subtilis DNA Polymerase III: Use in Enzyme Purification and Examination of Homology Among Replication-specific DNA Polymerases," Nucl. Acids Res., 6:1203-209 (1979); Barnes et al., "DNA Polymerase III of Mycoplasma pulmonis: Isolation and Characterization of the Enzyme and its Structural Gene, polC," Mol. Microb., 13:843-854, (1994); Low et al., "Purification and Characterization of DNA Polymerase III from *Bacillus subtilis*," J. Biol. Chem., 251:1311-1325 (1976)) instead of a 10 subunit assembly containing the three components of a rapidly processive machine as discussed above for Pol III holoenzyme from *E. coli*. This finding suggests a different structural organization of the replicase and possibly different functional characteristics as well.

Although there are many studies of replication mechanisms in eukaryotes and, specifically, the Gram negative bacterium *E. coli* and its bacteriophages, there is very little information about how Gram positive organisms replicate. The Gram positive class of bacteria includes some of the worst human pathogens such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Enterococcus faecalis*, and *Mycobacterium tuberculosis* (Youmans et al., The Biological and Clinical Basis of Infectious Disease (1985)). Until this invention, the best characterized Gram positive organism for chromosomal DNA synthesis was *Bacillus subtilis*. Fractionation of *B. subtitis* has identified three DNA polymerases. (Gass et al., "Further Genetic and Enzymological Characterization of the Three *Bacillus subtilis* Deoxyribonucleic Acid Polymerases," J. Biol. Chem., 248:7688-7700 (1973); Ganesan et al., "DNA Replication in a Polymerase I Deficient Mutant and the Identification of DNA Polymerases II and III in *Bacillus subtilis*," Biochem. Biophys. Res. Commun., 50:155-163 (1973)). These polymerases are thought to be analogous to the three DNA polymerases of *E. coli* (DNA polymerases I, II, and III). Studies in *B. subtilis* have identified a polymerase that appears to be involved in chromosome replication and is termed Pol III (Ott et al., "Cloning and Characterization of the *polC* Region of *Bacillus subtilis*," J. Bacteriol., 165:951-957 (1986); Barnes et al.,

"Localization of the Exonuclease and Polymerase Domains of *Bacillus subtilis* DNA Polymerase III," Gene, 111:43-49 (1992); Barnes et al., "The 3'-5' Exonuclease Site of DNA Polymerase III From Gram-positive Bacteria: Definition of a Novel Motif Structure," Gene" 165:45-50 (1995) or Barnes et al., "Purification of DNA Polymerase III of Gram-positive Bacteria," Methods in Enzy., 262:35-42 (1995)). The *B. subtilis* Pol III (encoded by *polC*) is larger (about 165 kDa) than the *E. coli* alpha subunit (about 129 kDa) and exhibits 3'-5' exonuclease activity. The *polC* gene encoding this Pol III shows weak homology to the genes encoding *E. coli* alpha and the *E. coli* epsilon subunit. Hence, this long form of the *B. subtilis* Pol III (herein referred to as α -large or Pol III-L) essentially comprises both the alpha and epsilon subunits of the *E. coli* core polymerase. The *S. aureus* α -large has also been sequenced, expressed in *E. coli,* and purified; it contains DNA polymerase and 3'-5' exonuclease activity (Pacitti et al., "Characterization and Overexpression of the Gene Encoding *Staphylococcus aureus* DNA Polymerase III," Gene, 165:51-56 (1995)). Although α -large is essential to cell growth (Clements et al., "Inhibition of *Bacillus subtilis* Deoxyribonucleic Acid Polymerase III by Phenylhydrazinopyrimidines: Demonstration of a Drug-induced Deoxyribonucleic Acid-Enzyme Complex," J. Biol. Chem., 250:522-526 (1975); Cozzarelli et al., "Mutational Alteraction of *Bacillus subtilis* DNA Polymerase III to Hydroxyphenylazopyrimidine Resistance: Polymerase III is Necessary for DNA Replication," Biochem. And Biophy. Res. Commun., 51:151-157 (1973); Low et al., "Mechanism of Inhibition of *Bacillus subtilis* DNA Polymerase III by the Arylhydrazinopyrimidine Antimicrobial Agents," Proc. Natl. Acad. Sci. USA, 71:2973-2977 (1974)), there could still be another DNA polymerase(s) that is essential to the cell, such as occurs in yeast (Morrison et al., "A Third Essential DNA Polymerase in *S. cerevisiae*," Cell, 62:1143-1151 (1990)).

Purification of α -large from *B. subtilis* results in only this single protein without associated proteins (Barnes et al., "Localization of the Exonuclease and Polymerase Domains of *Bacillus subtilis* DNA Polymerase III," Gene, 111:43-49 (1992); Barnes et al., "The 3'-5' Exonuclease Site of DNA Polymerase III From Gram-positive Bacteria: Definition of a Novel Motif Structure," Gene" 165:45-50 (1995) or Barnes et al., "Purification of DNA Polymerase III of Gram-positive Bacteria," Methods in Enzymol., 262:35-42 (1995)). Hence, it is possible that α -large is a member of the Type I replicase (like T5) in which it is processive on its own

without accessory proteins. *B. subtilis* and *S. aureus* also have a gene encoding a protein that has approximately 30% homology to the beta subunit of *E. coli*; however, the protein product has not been purified or characterized (Alonso et al., "Nucleotide Sequence of the recF Gene Cluster From *Staphylococcus aureus* and Complementation Analysis in *Bacillus subtilis recF* Mutants," Mol. Gen. Genet., 246:680-686 (1995); Alonso et al., "Nucleotide Sequence of the recF Gene Cluster From *Staphylococcus aureus* and Complementation Analysis in *Bacillus subtilis recF* Mutants," Mol. Gen. Genet., 248:635-636 (1995)). Whether this beta subunit has a function in replication, a ring shape, or functions as a sliding clamp was not known until recently. It was also not known whether it is functional with α -large. Recently, it was shown that *S. aureus* β is functional as a ring, and that it also functions with α -large (U.S. Patent Application Serial No. 09/235,245, which is hereby incorporated by reference). Further, a fourth DNA polymerase was identified with greater homology to *E. coli* α than α -large. This polymerase, called herein α -small, is shorter than α -large and lacks the domain homologous to epsilon. This polymerase also functions with the β ring, indicating that it may participate in chromosome replication. Indeed, a recent report indicates that α -small is essential for replication in *Streptomyces coelicolor* A3(2) (Flett et al., "A Gram-negative type' DNA Polymerase III is Essential for Replication of the Linear Chromosome of *Streptomyces Coelicolor* A3(2)," Mol. Micro., 31:949-958, (1999)).

As described earlier, purification of the replicase from the Gram positive *B. subtilis* gives only a single subunit Pol III, instead of a multicomponent complex. Also, *S. aureus dnaX* has been shown to encode only one subunit (U.S. Patent Application Serial No. 09/235,245, which is hereby incorporated by reference). Moreover, *S. aureus* and *B. subtilis* lack homologues to χ, ψ, θ, and the δ subunit is only weakly homologous to δ of *E. coli* (only 28%). Further, they lack a homologue to *dnaQ* encoding ε. Instead, they contain this activity (3'-5' exonuclease) in the *polC* gene product which provides the α -large form of α. The ε subunit is needed for high speed and processivity of the *E. coli* Pol III holoenzyme; the α subunit alone is much less rapid and processive with the β ring compared to the presence of both α and ε (Studwell et al., "Processive Replication is Contingent on the Exonuclease Subunit of DNA Polymerase III Holoenzyme," J. Biol Chem, 265: 1171-1178 (1990)).

Studies using the *E. coli* β ring (and γ complex) show they confer onto *S. aureus* α quite efficient synthesis (U.S. Patent Application Serial No. 09/235,245, which is hereby incorporated by reference), but the efficiency is not equal to that of *E. coli* αε with β (and γ complex). This may be due to use of the heterologous combination of an α subunit from one organism (*S. aureus*) with the β clamp from another (*E. coli*.). However, it is also possible that *S. aureus* α simply does not function with a β clamp to produce speed and processivity comparable to the *E. coli* polymerase. Also, as described earlier, the α -large subunit of *B. subtilis* purifies as a single subunit, rather than associated with accessory subunits assembled into the three components of a rapid, processive machine (i.e., like *E. coli* Pol III holoenzyme). The lack of two DnaX products, lack of a multicomponent structure, and lack of gene homologues encoding several subunits of the three component, Pol III, of *E. coli* brings into question whether other types of bacteria, such as Gram positive cells, even have an enzyme with similar structure or comparable speed and processivity to that found in the Gram negative *E. coli*.

The lack of gene homologues encoding several subunits of the *E. coli* three component polymerase creates uncertainties with respect to reconstructing a rapid and processive polymerase from a Gram positive cell that has characteristics like the Pol III system of *E. coli*.

The γ and δ' proteins are homologous to one another, encoding C-shape proteins (Dong et al., "DNA Polymerase III Accessory Proteins," J. Biol. Chem, 268:11758-11765, (1993); Guenther et al., "Crystal Structure of the δ' Subunit of the Clamp-loader Complex of *E. coli* DNA Polymerase III," Cell, 91:335-345 (1997)). The clamp loaders of yeast and humans are composed of five proteins, all of which are homologous to one another and to γ and δ' (Cullman et al., "Characterization of the Five Replication Factor C Genes of Saccharomyces Cerevisiae," Mol. Cell. Biol., 15:4661-4671 (1995)). This provides evidence that a clamp loader can be composed entirely of C-shape proteins. Perhaps the Gram positive DnaX-protein (hereafter referred to as τ) and δ' are sufficient to provide function as a clamp loader. Indeed, the clamp loader of T4 phage is composed of only two different proteins, gp44/62 complex (Young et al., "Structure and Function of the Bacteriophage T4 DNA Polymerase Holoenzyme," Biochem., 31:8675-8690 (1992)). This idea is also

supported by the presence of only two RFC genes in archaebacteria, suggesting that they may utilize two C-shaped proteins for clamp loading, in contrast to yeast and humans that use five. With this consideration in mind, genes were identified and isolated and the τ protein (encoded by *dnaX*) and δ' (encoded by *holB*) of another Gram positive organism, *Streptococcus pyogenes*, were expressed and purified. As was observed in *S. aureus*, *S. pyogenes dnaX* produces only a single polypeptide. The β, encoded by *dnaN* of *S. pyogenes*, was also identified, expressed, and purified, as were the α -large subunit encoded by *polC* and the SSB encoded by the *ssb* gene. These proteins were studied for interactions and characterized for their effect on α-large. However, the hypothesis was incorrect as τ and δ' did not form a τδ' complex, nor did they assemble β onto DNA or provide stimulation of α when using β on primed and SSB coated M13mp18 ssDNA.

In light of the inability of *S. pyogenes* τ protein and δ' to function as a clamp loader, it seemed reasonable to expect that one or more other proteins are needed. The fact that *E. coli* has some replicase subunits that other bacteria do not, suggests that other bacteria may have some replicase subunits that *E. coli* does not. Indeed, genetic studies of *Bacillus subtilis* demonstrates that it has three genes needed for replication that *E. coli* does not have. Two of these novel genes, called *dnaB* (not the same as *E. coli dnaB* encoding the helicase) and *dnaH*, have no significant homology to genes in the *E. coli* genome database (Bruand et al., "Nucleotide Sequence of the *Bacillus subtilis dnaD* gene," Microbiol., 141:321-322 (1995); Hoshino et al., "Nucleotide Sequence of *Bacillus subtilis dnaB*: A gene Essential for DNA replication Initiation and Membrane Attachment," Proc. Natl. Acad. Sci. USA, 84:653-657 (1987)). Further, *dnaI* of *B. subtilis* is important for replication and has, at best, a very limited homology to *E. coli dnaC* (Karamata et al., "Isolation and Genetic Analysis of Temperature-Sensitive Mutants of *B. subtilis* Defective in DNA synthesis," Molec. Gen. Genetics, 108:277-287 (1970)). Perhaps one or more of these genes encode the proteins(s) necessary to provide clamp loading activity when combined with τ and δ', or to couple with α to provide it with speed and/or processivity as the *E. coli* epsilon does. The *S. pyogenes* homologues of *B. subtilis* *dnaI*, *dnaH*, and *dnaB* were identified, cloned, and the encoded proteins were expressed and purified. However, these proteins failed to provide activity alone or in

combinations with *S. pyogenes* τ and δ' in loading *S. pyogenes* β onto DNA, or in stimulating *S. pyogenes* α -large in combination with β, τ, and δ' on SSB coated primed M13mp18 ssDNA.

Weak homology exists for the *holA* gene among prokaryotes. This weak homologue of *holA* was identified in *S. pyogenes* and, then, it was cloned, expressed, and the putative δ was purified. The putative δ formed an isolatable complex with τ and δ'. In fact, the τδδ' complex loaded *S. pyogenes* β onto DNA, and it stimulated *S. pyogenes* α -large in a β dependent reaction on primed SSB coated M13mp18 ssDNA. Hence, this protein was the only missing component necessary to provide clamp loading activity. Further, a mixture of α with τδδ', followed by ion exchange chromatography on MonoQ, indicated formation of an ατδδ' complex. Consistent with this, τ appeared to bind α in gel filtration analysis.

Whether the *S. pyogenes* three component polymerase can synthesize DNA in as rapid and processive of a fashion as the *E. coli* Pol III holoenzyme three component polymerase is very difficult to predict, because no other DNA polymerase known to date catalyzes synthesis at the rate or processivity of the *E. coli* three component polymerase. For example, the three component T4 phage polymerase travels about 400 nucleotides/s, the yeast DNA polymerase delta three component polymerase travels about 120 nucleotides/s, and the human DNA polymerase delta three component enzyme appears slower and less processive than the yeast enzyme.

The standard test for these speed and processivity characteristics is examination of a time course in extension of a primer on a very long template, such as around the 7.2 kb M13mp18 ssDNA genome coated with SSB and primed with a synthetic DNA oligonucleotide. The results of experiments of this type demonstrate that the three component *S. pyogenes* polymerase is indeed extremely rapid in synthesis. Surprisingly, it is just as fast as the *E. coli* enzyme. Extension proceeds at about 700-800 nucleotides per second, completing the entire template in about 9 seconds. The enzyme was fully processive throughout replication of the M13mp18 genome, as could be determined from the fact that some templates were not extended at all, while others were extended to completion. If the enzyme had not been processive during the entire replication reaction, then when it comes off one partially extended DNA genome it would have reassociated with the unextended DNA that

remained and partially replicated it as well (and so on until the entire population of DNA became fully replicated). This did not happen. Instead, the reaction showed a mixture of completely replicated templates and templates that were still untouched starting material. This indicates that the enzyme stays with a template until it completes it before it cycles over to replicate another one (i.e., it is highly processive). Each of the five proteins, α, τ, δ, δ' and β, are needed to obtain this rapid and processive DNA synthesis.

This invention has provided an intellectual template by which the clamp loader component of these three component polymerases can be obtained from any eubacterial prokaryotic cell and how to use it with the other components to produce a rapid and processive polymerase. All prokaryotes in the eubacterial kingdom that have been sequenced to date contain homologues of these genes. As the process of lateral gene transfer appears to be a major force in evolution, it would appear that relatedness of enzymes and enzyme machines is best judged by comparisons of their genes and proteins rather than by phylogeny of which bacteria they are in (Doolittle et al., "Archaeal Genomics: Do Archaea have a Mixed Heritage?," Curr. Biol., 8:R209-R211 (1998)). As pointed out earlier in this application, most bacteria have genetic characteristics of replication genes/proteins of *S. pyogenes* rather than that of *E. coli* (i.e., no genes encoding χ,ψ, or θ, only a weak homolog to δ, or a *dnaX* gene encoding only a single protein).

The *dnaX* gene encoding τ and γ in *E. coli* encodes only one protein in some organisms, but, as this application shows, it is still functional in forming a protein complex capable of rapid and processive DNA synthesis. In addition, this application shows that the delta subunit, which is only weakly homologous among different prokaryotic organisms, is an essential functional subunit of the three component polymerase (instead of having diverged so as to fulfill an entirely different function in some other intracellular process). As mentioned earlier, several genes encoding subunits of the *E. coli* clamp loader (γ complex; γ,δ,δ',χ,ψ) are not obviously present in other prokaryotes (*holC* and *holD* encoding χ and ψ). Hence, one may anticipate that other genes may have evolved to encode new subunits that replace these, and that these new subunits may have been essential to the activity of the clamp loader. For example, they may have either taken over some of the functionality of

another subunit, or structurally (e.g., the physical presence of a subunit could be needed for one subunit to assume its proper and active conformation, or for one or more of the subunits to form a complex together to yield the multisubunit clamp loader assembly). In addition, this application shows that the α subunit (*polC* gene product) is sufficient for rapid and processive synthesis with the other two components (i.e., *E. coli* requires ε submit to bind to α for rapid and processive synthesis of α with the β clamp). Finally, this application shows that the *S. pyogenes* three component polymerase synthesizes DNA as fast as the *E. coli* Pol III three component polymerase. Up to this point, the *E. coli* Pol III three component polymerase was over twice the speed of the T4 enzyme and over 5 times the speed of others. Hence, it was possible that *E. coli* may have been unique among prokaryotes in having a polymerase that achieves such speed. This invention shows that this is not the case. Instead, this speed in polymerization generalizes to the Gram positive prokaryotic three component DNA polymerases. It may be presumed, now that two examples of three component polymerases in widely divergent bacteria share the charactistics of rapid, processive synthesis, that the three component polymerase of other eubacteria will also be rapid and processive.

These rapid and processive three component DNA polymerases can be applied to several important uses. DNA polymerases currently in use for DNA sequencing and DNA amplification use enzymes that are much slower and thus could be improved upon. This is especially true of amplification as the three component polymerase is capable of speed and high processivity making possible amplification of very long (tens of Kb to Mb) lengths of DNA in a time efficient manner. These three component polymerases also function in conjunction with a replicative helicase (DnaB) and, thus, are capable of amplification at ambient temperature using the helicase to melt the DNA duplex. This property could be useful in amplification reaction procedures such as in polymerase chain reaction (PCR) methodology. Finally, these three component polymerases and their associated helicase (DnaB) and primase (DnaG) are attractive targets for antibiotics due to their essential and central role in cell viability.

This application provides a three component polymerase from two human pathogens in the Gram positive class. It makes possible the production of this three component polymerase from other bacteria of the Gram positive type (e.g.,

Streptococci, *Staphylococci*, *Mycoplasma*) and other types of bacteria lacking χ,ψ, or θ, those having only one protein produced by their *dnaX* gene such as obligate intracellular parasites, Mycoplasmas (possibly evolved from Gram positives), Cyanobacteria (*Synechocystis)*, Spirochaetes such as *Borrelia* and *Treponemia* and *Chlamydia*, and distant relatives of *E. coli* in the Gram negative class (e.g., *Rickettsia* and *Helicobacter*). These three component polymerases are useful in manipulation of nucleic acids for research and diagnostic purposes (e.g., sequencing and amplification methods) and for screening chemicals for antibiotic activity (useful in human or animal therapy and agriculture such as animal feed supplements). There are several assays described previously in U.S. Patent Application Serial No. 09/235,245 to O'Donnell et al., which is hereby incorporated by reference, that use these three component polymerases (or subassemblies), as well as the DnaB and DnaG homologues, either alone or in various combinations, for the purpose of screening chemicals, such as chemical libraries, for inhibitor activity. Such inhibitors can be developed further (usually by chemical manipulation and alteration) into lead compounds and then into full fledged pharmaceuticals.

There remains a need to understand the molecular details of the process of DNA replication in other cells that are quite different from *E. coli*, such as in Gram positive cells. It is possible that a more detailed understanding of replication proteins will lead to discovery of new antibiotics. Therefore, a deeper understanding of replication proteins of Gram positive bacteria is especially important given the emergence of drug resistant strains of these organisms. For example, *Staphylococcus aureus* has successfully mutated to become resistant to all common antibiotics.

The "target" protein(s) of an antibiotic drug is generally involved in a critical cell function, such that blocking its action with a drug causes the pathogenic cell to die or no longer proliferate. Current antibiotics are directed to very few targets. These include membrane synthesis proteins (e.g., vancomycin, penicillin, and its derivatives such as ampicillin, amoxicillin, and cephalosporin), the ribosome machinery (e.g., tetracycline, chloramphenicol, azithromycin, and the aminoglycosides such as kanamycin, neomycin, gentamicin, streptomycin), RNA polymerase (e.g., rifampimycin), and DNA topoisomerases (e.g., novobiocin, quinolones, and fluoroquinolones). The DNA replication apparatus is a crucial life process and, thus, the proteins involved in this process are good targets for antibiotics.

A powerful approach to discovery of a new drug is to obtain a target protein, characterize it, and develop *in vitro* assays of its cellular function. Large chemical libraries can then be screened in the functional assays to identify compounds that inhibit the target protein. These candidate pharmaceuticals can then be chemically modified to optimize their potency, breadth of antibiotic spectrum, non-toxicity, performance in animal models and, finally, clinical trials. The screening of large chemical libraries requires a plentiful source of the target protein. An abundant supply of protein generally requires overproduction techniques using the gene encoding the protein. This is especially true for replication proteins as they are present in low abundance in the cell.

Selective and robust assays are needed to screen reliably a large chemical library. The assay should be insensitive to most chemicals in the concentration range normally used in the drug discovery process. These assays should also be selective and not show inhibition by antibiotics known to target proteins in processes outside of replication.

The present invention is directed to overcoming these deficiencies in the art.

SUMMARY OF THE INVENTION

The present invention relates to various isolated DNA molecules from *Staphylococcus aureus* and *Streptococcus pyogenes*, both of which are Gram positive bacteria. These include DNA molecules which include a coding region from the *dnaE* gene (encoding α- small), *dnaX* gene (encoding tau), *polC* gene (encoding Pol III –L or α- large), *dnaN* gene (encoding beta), *holA* gene (encoding delta), *holB* gene (encoding delta prime), *ssb* gene (encoding SSB), *dnaB* gene (encoding DnaB), and *dnaG* gene (encoding DnaG) of *S. aureus* and/or *S. pyogenes*. These DNA molecules can be inserted into an expression system and used to transform host cells. The isolated proteins or polypeptides encoded by these DNA molecules, and their ability to function when used in combination is also disclosed. The resulting actions provide assembling a ring onto DNA via a clamp loader, and polymerase activity dependent on this ring that is rapid and processive.

A further aspect of the present invention relates to a method of identifying compounds which inhibit activity of a polymerase product of *polC* or *dnaE*. This method is carried out by forming a reaction mixture comprising a primed DNA molecule, a polymerase product of *polC* or *dnaE*, a candidate compound, a dNTP, and optionally either a beta subunit, a tau complex, or both the beta subunit and the tau complex, wherein at least one of the polymerase product of *polC* or *dnaE*, the beta subunit, the tau complex, or a subunit or combination of subunits thereof is derived from a Eubacteria other than *Escherichia coli*; subjecting the reaction mixture to conditions effective to achieve nucleic acid polymerization in the absence of the candidate compound; analyzing the reaction mixture for the presence or absence of nucleic acid polymerization extension products; and identifying the candidate compound in the reaction mixture where there is an absence of nucleic acid polymerization extension products.

The present invention deciphers the structure and mechanism of the chromosomal replicase of Gram positive bacteria and other bacteria lacking *holC, holD, holE* or *dnaQ* genes, or having a *dnaX* gene that encodes only one protein. Rather than use a DNA polymerase that attains high efficiency on its own, or with one other subunit, the Gram positive bacteria replicase is a three component type of replicase (class III) that uses a sliding clamp protein. The Gram positive bacteria replicase also uses a clamp loader component that assembles the sliding clamp onto DNA. This knowledge, and the enzymes involved in the replication process, can be used for the purpose of screening for potential antibiotic drugs. Further, information about chromosomal replicases may be useful in DNA sequencing, DNA amplification, polymerase chain reaction, and other DNA polymerase related techniques.

The present invention identifies two DNA polymerases (both of Pol III type) in Gram positive bacteria that utilize the sliding clamp and clamp loader. The present invention also identifies a gene with homology to the alpha subunit of *E. coli* DNA polymerase III holoenzyme, the chromosomal replicase of *E. coli*. These DNA polymerases can extend a primer around a large circular natural template when the beta clamp has been assembled onto the primed ssDNA by the clamp loader or a primer on a linear DNA where the beta clamp may assemble by itself by sliding over an end.

The present invention shows that the clamp and clamp loader components of Gram negative cells can be exchanged for those of Gram positive cells in that the clamp, once assembled onto DNA, will function with Pol III obtained from either Gram positive and Gram negative sources. This result implies that important contacts between the polymerase and clamp have been conserved during evolution. Therefore, these "mixed systems" may provide assays for an inhibitor of this conserved interaction. Such an inhibitor may be expected to shut down replication, and since the interaction is apparently conserved across the evolutionary spectrum from Gram positive and Gram negative cells, the inhibitor may exhibit a broad spectrum of antibiotic activity.

The present invention demonstrates that Gram positive bacteria contain a beta subunit that behaves as a sliding clamp that encircles DNA. A *dnaX* gene sequence encoding a protein homolog of the gamma/tau subunit of the clamp loader (gamma/tau complex) *E. coli* DNA polymerase III holoenzyme is also identified. The presence of this gene confirms the presence of a clamp loading apparatus in Gram positive bacteria that will assemble beta clamps onto DNA for the DNA polymerases.

This application also outlines methods and assays for use of these replication proteins in drug screening processes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of the *S. aureus* Pol III-L expression vector. The gene encoding Pol III-L was cloned into a pET11 expression vector in a three step cloning scheme as illustrated.

Figures 2A-C describe the expression and purification of *S. aureus* Pol III-L (alpha-large). Figure 2A compares *E. coli* cells that contain the pET11PolC expression vector that are either induced or uninduced for protein expression. The gel is stained with Coomassie Blue. The induced band corresponds to the expected molecular weight of the *S. aureus* Pol III-L, and is indicated to the right of the gel. Figure 2B shows the results of the MonoQ chromatography of a lysate of *E. coli* (pET11PolC-L) induced for Pol III-L. The fractions were analyzed in a Coomassie Blue stained gel (top) and for DNA synthesis (bottom). Fractions containing Pol III-L are indicated. In Figure 2C, fractions containing Pol III-L from the MonoQ column

were pooled and chromatographed on a phosphocellulose column. This shows an analysis of the column fractions from the phosphocellulose column in a Coomassie Blue stained polyacrylamide gel. The position of Pol III-L is indicated to the right.

Figure 3 shows the *S. aureus* beta expression vector. The *dnaN* gene was amplified from *S. aureus* genomic DNA and cloned into the pET16 expression vector.

Figures 4A-C illustrate the expression and purification of *S. aureus* beta. Figure 4A compares *E. coli* cells that contain the pET16beta expression vector that are either induced or uninduced for protein expression. The gel is stained with Coomassie Blue. The induced band corresponds to the expected molecular weight of the *S. aureus* beta, and is indicated to the right of the gel. The migration position of size standards are indicated to the left. Figure 4B shows the results of MonoQ chromatography of an *E. coli* (pET16beta) lysate induced for beta. The fractions were analyzed in a Coomassie Blue stained gel, and fractions containing beta are indicated. In Figure 4C, fractions containing beta from the MonoQ column were pooled and chromatographed on a phosphocellulose column. This shows an analysis of the column fractions from the phosphocellulose column in a Coomassie Blue stained polyacrylamide gel. The position of beta is indicated to the right.

Figures 5A-B demonstrate that the *S. aureus* beta stimulates *S. aureus* Pol III-L and *E. coli* Pol III core on linear DNA, but not circular DNA. In Figure 5A, the indicated proteins were added to replication reactions containing polydA-oligodT as described in the Examples *infra*. Amounts of proteins added, when present, were: lanes 1,2: *S. aureus* Pol III-L, 7.5 ng; *S. aureus* beta, 6.2 μg; Lanes 3,4: *E. coli* Pol III core, 45 ng; *S. aureus* beta, 9.3 μg; Lanes 5,6: *E. coli* Pol III core, 45 ng; *E. coli* beta, 5μg. Total DNA synthesis was: Lanes 1-6: 4.4, 30.3, 5.1, 35.5, 0.97, 28.1 pmol, respectively. In Figure 5B, Lanes 1-3, the indicated proteins were added to replication reactions containing circular singly primed M13mp18 ssDNA as described in the Examples *infra*. *S. aureus* beta, 0.8 μg; *S. aureus* Pol III-L, 300 ng (purified through MonoQ); *E. coli* clamp loader complex, 1.7 μg. Results in the *E. coli* system are shown in Lanes 4-6. Total DNA synthesis was: Lanes 1-6: 0.6, 0.36, 0.99, 2.7, 3.5, 280 pmol, respectively.

Figure 6 shows that *S. aureus* Pol III-L functions with *E. coli* beta and clamp loader complex on circular primed DNA. It also shows that *S. aureus* beta does

not convert Pol III-L with sufficient processivity to extend the primer all the way around a circular DNA. Replication reactions were performed on the circular singly primed M13mp18 ssDNA. Proteins added to the assay are as indicated in this figure. The amount of each protein, when present, is: *S. aureus* beta, 800 ng; *S. aureus* Pol III-L, 1500 ng (MonoQ fraction 64); *E. coli* Pol III core, 450 ng; *E. coli* beta, 100 ng; *E. coli* gamma complex, 1720 ng. Total DNA synthesis in each assay is indicated at the bottom of the figure.

Figures 7A-B show that *S. aureus* contains four distinct DNA polymerases. Four different DNA polymerases were partially purified from *S. aureus* cells. *S. aureus* cell lysate was separated from DNA and, then, chromatographed on a MonoQ column. Fractions were analyzed for DNA polymerase activity. Three peaks of activity were observed. The second peak was the largest and was expected to be a mixture of two DNA polymerases based on early studies in *B. subtilis*. Chromatography of the second peak on phosphocellulose (Figure 7B) resolved two DNA polymerases from one another.

Figures 8A-B show that *S. aureus* has two DNA Pol III's. The four DNA polymerases partially purified from *S. aureus* extract, designated peaks I-IV in Figure 7, were assayed on circular singly primed M13mp18 ssDNA coated with *E. coli* SSB either in the presence or absence of *E. coli* beta (50ng) and clamp loader complex (50 ng). Each reaction contained 2 µl of the partially pure polymerase (Peak 1 was Mono Q fraction 24 (1.4 µg), Peak 2 was phosphocellulose fraction 26 (0.016 mg/ml), Peak 3 was phosphocellulose fraction 46 (0.18 mg/ml), and Peak 4 was MonoQ fraction 50 (1 µg). Figure 8A shows the product analysis in an agarose gel. Figure 8B shows the extent of DNA synthesis in each assay.

Figure 9 compares the homology between the polypeptide encoded by *dnaE* of *S. aureus* and other organisms. An alignment is shown for the amino acid sequence of the *S. aureus dnaE* product with the *dnaE* products (alpha subunits) of *E. coli* and *Salmonella typhimurium*.

Figure 10 compares the homology between the N-terminal regions of the gamma/tau polypeptides of *S. aureus*, *B. subtilis*, and *E. coli*. The conserved ATP site and the cystines forming the zinc finger are indicated above the sequence. The organisms used in the alignment were: *E. coli* (GenBank); and *B. subtilis*.

Figure 11 compares the homology between the DnaB polypeptide of *S. aureus* and other organisms. The organisms used in the alignment were: *E. coli* (GenBank); *B. subtilis*; *Sal.Typ.*, (*Salmonella typhimurium*).

Figures 12A-B show the alignment of the delta subunit encoded by *holA* for *E. coli* and *B. subtilis* (Figure 12A) and for the delta subunit of *B. subtilis* and *S. pyogenes* (Figure 12B). Figure 12A shows ClustalW generated alignment of *S. pyogenes* (Gram positive) delta to *E.coli* (Gram negative) delta. Figure 12B shows ClustalW generated alignment of *B. subtilis* (Gram positive) delta to *S. pyogenes* (Gram positive) delta.

Figure 13 is an image of an autoradiograph of an agarose gel analysis of replication products from singly primed, SSB coated M13mp18 ssDNA using the reconstituted *S. aureus* Pol III holozyme. Only in the presence of the τδδ' complex does α-large (PolC) function with β to replicate a full circular duplex DNA (RFII).

Figure 14 shows a Comassie Blue stained SDS polyacrylamide gel of the pure *S. pyogenes* subunits corresponding to alpha-large, alpha-small, *dnaX* gene product (called tau), beta, delta, delta prime, and SSB. The first lane shows the position of molecular weight markers. Purified proteins were separated on a 15% SDS-PAGE and stained with Coommassie Brilliant Blue R-250. Each lane contains 5 microgram of each protein. Lane 1, markers; lane 2, alpha-large; lane 3, alpha-small, lane 4, tau subunit; lane 5, beta subunit; lane 6, delta subunit; lane 7, delta prime subunit; lane 8, single strand DNA binding protein.

Figures 15A-C document the ability to reconstitute the τδδ' complex of *S. pyogenes*. Proteins were mixed and gel filtered on Superose 6, followed by analysis of the column fractions in a SDS polyacrylamide gel. Figure 15A shows a mixture of τδδ'. Figure 15B shows a mixture of τδ. Figure 15C shows a mixture of τδ'.

Figures 16A-E show that the *S. pyogenes* τδδ' complex can load the *S. pyogenes* beta clamp onto (circular) DNA. Loading reactions contained 500 fm nicked pBSK plasmid, 500 fm either τδδ' complex, tau, delta, or delta prime, 1pm ^{32}P-labelled beta dimer, 8 mM $MgCl_2$, 1 mM ATP. Reaction components were preincubated for 10 min at 37°C prior to loading onto 5 ml Biogel A15M column equilibrated with buffer A containing 100 mM NaCl. Figure 16A demonstrates the ability of τδδ' complex to load the beta dimer onto a nicked pBSK circular plasmid.

Figures 16B-E show the results of using either: beta alone (Figure 16B); δδ' plus β (Figure 16C); τ, δ and β (Figure 16D); τ, δ' and β (Figure 16E).

Figures 17A-C show that τ and alpha interact. Figure 17A shows the result of gel filtration analysis of a mixture of τ with alpha-large. Gel filtration fractions are analyzed in a SDS polyacrylamide gel. Figures 17B and 17C show the results using only τ or only alpha-large, respectively. Comparison of the elution positions of proteins shows that the positions of alpha and tau are shifted toward a higher molecular weight complex when they are present together. The fact they do not exactly comigrate may indicate that they initially are together in a complex, but that the complex dissociates during the time of the gel filtration experiment (over one half hour).

Figures 18A-B document the ability to reconstitute $\alpha_L\tau\delta\delta'$ (pol III*) complex of *S. pyogenes*. Proteins were mixed, preincubated for 20 min at 15°C, gel filtered on Superose 6, followed by analysis of the column fractions in a SDS polyacrylamide gel (Figure 18A). Proteins were loaded on a MonoQ column, then eluted with a linear gradient of 50-500 mM NaCl, followed by analysis of the column fractions in a SDS polyacrylamide gel (Figure 18B). The $\alpha_L\tau\delta\delta'$ complex migrates early.

Figure 19 illustrates the speed and processivity of the *S. pyogenes* $\alpha_L\tau\delta\delta'$ (pol III*) complex. The $\alpha_L\tau\delta\delta'$ (pol III*) complex was incubated with primed M13pm18 ssDNA (coated with *S. pyogenes* SSB) and only two dNTPs, then replication was initiated upon adding the remaining two dNTPs. Reactions contained 25 fmol singly primed M13mp18 ssDNA template, 300 fmol β_2, and either 75 fmol or 250 fmol $\alpha_L\tau\delta\delta'$. Time points were quenched with SDS/EDTA then analyzed in a neutral agarose gel followed by autoradiography. Each time point is a separate reaction. The time course of polymerization was performed at two different ratios of polymerase/primed template to assess speed and processivity of nucleotide incorporation.

Figures 20A-I show the extent of homology between *S. pyogenes* replication genes and other organisms. Due to the low homology of delta (Figure 20D), one must "walk" from one organism to the next in order to recognize the homologue with high probability. Percent identity over regions of the indicated

number of amino acid residues is shown for each match (i.e., the two organisms at the opposite ends of each line). Amino acid sequences were retrieved from either GenBank or individual unfinished genome databases.

Figure 21A-F are images illustrating that the *S. pyogenes* DnaE (alpha-small) polymerase functions with β. Figures 21A-B illustrate the relationship between DnaE and β for association with ssDNA. Different amounts of DnaE polymerase were added to a SSB coated M13mp18 ssDNA circle primed with a single DNA oligonucleotide, and products were analyzed in a native agarose gel. Reactions were performed in the presence of τδδ' and either the absence (Figure 21C, panels 1-4) or presence (Figure 21D, panels 1-4) of β. Positions of completed duplex (RFII) and initial primed template (ssDNA) are indicated. Figure 21E shows an analysis of exonuclease activity by PolC and DnaE on a 5'-32P-DNA 30-mer. Aliquots were removed at the indicated times and analyzed in a sequencing gel. Figure 21F shows the effect of TMAU on PolC and DnaE in the presence of τδδ' and β. DNA products were analyzed in a native agarose gel. Positions of initial primed M13mp18 (ssDNA) and completed circular duplex (RFII) are indicated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to various isolated nucleic acid molecules from Gram positive bacteria and other bacteria lacking *holC, holD,* or *holE* genes or having a *dnaX* gene encoding only one subunit. These include DNA molecules which correspond to the coding regions of the *dnaE, dnaX, holA, holB, polC, dnaN, SSB, dnaB,* and *dnaG* genes. These DNA molecules can be inserted into an expression system or used to transform host cells. The isolated proteins or polypeptides encoded by these DNA molecules and their use to form a three component polymerase are also disclosed. Also encompassed by the present invention are corresponding RNA molecules transcribed from the DNA molecules.

These DNA molecules and proteins can be derived from numerous bacteria, including *Staphylococcus, Streptococcus, Enterococcus, Mycoplasma, Mycobacterium, Borrelia, Treponema, Rickettsia, Chlamydia, Helicobacter,* and *Thermatoga*. It is particularly directed to such DNA molecules and proteins derived from *Streptococcus* and *Staphylococcus* bacteria, particularly *Streptococcus pyogenes*

and *Staphylococcus aureus* (see U.S. Patent Application Serial No. 09/235,245, which is hereby incorporated by reference).

The gene sequences used to obtain DNA molecules of the present invention were obtained by sequence comparisons with the *E. coli* counterparts, followed by detailed analysis of the raw sequence data in the contigs from the *S. pyogenes* database (http://dna1.chem.ou.edu/strep.html) or the *S. aureus* database (http://www.genome.ou.edu/staph.html) to identify the open reading frames. In many instances, nucleotide errors were observed causing frameshifts in the open reading frame thus truncating it. Therefore, upon cloning the genes via PCR, the genes were sequenced to obtain correct information. Also, the full nucleotide sequence of the *ssb* gene was not present in the data base. This was cloned by circular PCR and the full sequence is reported below.

The *S. aureus dnaX* and *dnaE* genes were identified by aligning genes of several organisms and designing primers for use in PCR to obtain a gene fragment, followed by steps to identify the entire gene.

One aspect of the present invention relates to a newly discovered Pol III gene (herein identified as *dnaE*) of *S. aureus* whose encoded protein is homologous to *E. coli* alpha (product of *dnaE* gene). The partial nucleotide sequence of the *S. aureus dnaE* gene corresponds to SEQ. ID. No. 1 as follows:

```
atggtggcat atttaaatat tcatacggct tatgatttgt taaattcaag cttaaaaata 60
gaagatgccg taagacttgc tgtgtctgaa aatgttgatg cacttgccat aactgacacc 120
aatgtattgt atggttttcc taaattttat gatgcatgta tagcaaataa cattaaaccg 180
atttttggta tgacaatata tgtgacaaat ggattaaata cagtcgaaac agttgttcta 240
gctaaaaata atgatggatt aaaagatttg tatcaactat catcggaaat aaaaatgaat 300
gcattagaac atgtgtcgtt tgaattatta aaacgatttt ctaacaatat gattatcatt 360
tttaaaaaag tcggtgatca acatcgtgat attgtacaag tgtttgaaac ccataatgac 420
acatatatgg accaccttag tatttcgatt caaggtagaa aacatgtttg gattcaaaat 480
gtttgttacc aaacacgtca agatgccgat acgatttctg cattagcagc tattagagac 540
aatacaaaat tagacttaat tcatgatcaa gaagattttg gtgcacattt tttaactgaa 600
aaggaaatta atcaattaga tattaaccaa gaatatttaa cgcaggttga tgttatagct 660
caaaagtgtg atgcagaatt aaaatatcat caatctctac ttcctcaata tgagacacct 720
aatgatgaat cagctaaaaa atatttgtgg cgtgtcttag ttacacaatt gaaaaaatta 780
gaacttaatt atgacgtcta tttagagcga ttgaaatatg agtataaagt tattactaat 840
atgggttttg aagattattt cttaatagta agtgatttaa tccattatgc gaaaacgaat 900
gatgtgatgg taggtcctgg tcgtggttct tcagctggct cactggtcag ttatttattg 960
ggaattacaa cgattgatcc tattaaattc aatctattat ttgaacgttt tttaaaccca 1020
gaacgtgtaa caatgcctga tattgatatt gactttgaag atacacgccg agaaagggtc 1080
attcagtacg tccaagaaaa atatggcgag ctacatgtat ctggaattgt gactttcggt 1140
catctgcttg caagagcagt tgctagagat gttggaagaa ttatggggtt tgatgaagtt 1200
acattaaatg aaatttcaag tttaatccca cataaattag gaattacact tgatgaagca 1260
tatcaaattg acgattttaa agagtttgta catcgaaacc atcgacatga acgctggttc 1320
agtatttgta aaaagttaga aggtttacca agacatacat ctacacatgc ggcaggaatt 1380
attattaatg accatccatt atatgaatat gccccctttaa cgaaagggga tacaggatta 1440
ttaacgcaat ggacaatgac tgaagccgaa cgtattgggt tattaaaaat agattttcta 1500
gggttgagaa acttatcgat tattcatcaa atcttaacac aagtcaaaaa agatttaggt 1560
```

```
attaatattg atatcgaaaa gattccgttt gatgatcaaa aagtgtttga attgttgtcg 1620
caaggagata cgactggcat attccaatta gagtctgacg gtgtaagaag tgtattaaaa 1680
aaattaaagc cggaacactt tgaagatatt gttgctgtaa cttctttgta tagaccaggt 1740
ccaatggaag aaattccaac ttacattaca agaagacatg atccaagcaa agttcaatat 1800
ttacatccgc atttagaacc tatattaaaa aatacttacg gtgttattat ttatcaagag 1860
caaattatgc aaatagcgag cacatttgca aacttcagtt atggtgaagc ggatatttta 1920
agaagagcaa tgagtaaaaa aaatagagct gttcttgaaa gtgagcgtca acattttata 1980
gaaggtgcaa agcaaaatgg ttatcacgaa gacattagta agcaaatatt tgatttgatt 2040
ctgaaatttg ctgattatgg ttttcctaga gcacatgctg tcagctattc taaaattgca 2100
tacattatga gctttttaaa agtccattat ccaaattatt tttacgcaaa tattttaagt 2160
aatgttattg gaagtgagaa gaaaactgct caaatgatag aagaagcaaa aaaacaaggt 2220
atcactatat tgccaccgaa cattaacgaa agtcattggt tttataaacc ttcccaagaa 2280
ggcatttatt tatcaattgg tacaattaaa ggtgttggtt atcaaagtgt gaaagtgatt 2340
gttgatgaac gttatcagaa cggcaaattt aaagatttct ttgattttgc tagacgtata 2400
ccgaagagag tcaaaacgag aaagttactt gaagcactga ttttagtggg agcgtttgat 2460
gcttttggta aaacacgttc aacgttgttg caagctattg atcaagtgtt ggatggcgat 2520
ttaaacattg aacaagatgg ttttttattt gatattttaa cgccaaaaca gatgtatgaa 2580
gataaagaag aattgcctga tgcacttatt agtcagtacg aaaaagaata tttaggattt 2640
tatgtttcgc aacacccagt agataaaaag tttgttgcca aacaatattt aacgatattt 2700
aaattgagta acgcgcagaa ttataaacct atattagtac agtttgataa agttaaacaa 2760
attcgaacta aaaatggtca aaatatggca ttcgtcacat taaatgatgg cattgaaact 2820
ttagatggtg tgattttccc taatcagttt aaaaagtacg aagagttgtt atcacataat 2880
gacttgttta tagttagcgg gaaatttgac catagaaagc aacaacgtca actaattata 2940
aatgagattc agacattagc cacttttgaa gaacaaaaat tagcatttgc caaacaaatt 3000
ataattagaa ataaatcaca aatagatatg tttgaagaga tgattaaagc tacgaaagag 3060
aatgctaatg atgttgtgtt atccttttat gatgaaacga ttaaacaaat gactacttta 3120
ggctatatta atcaaaaaga tagtatgttt aataatttta tacaatcctt taaccctagt 3180
gatattaggc ttata                                                  3195
```

The *S. aureus dnaE* encoded protein, called α-small, has an amino acid sequence corresponding to SEQ. ID. No. 2 as follows:

```
Met Val Ala Tyr Leu Asn Ile His Thr Ala Tyr Asp Leu Leu Asn Ser
 1               5                  10                  15

Ser Leu Lys Ile Glu Asp Ala Val Arg Leu Ala Val Ser Glu Asn Val
            20                  25                  30

Asp Ala Leu Ala Ile Thr Asp Thr Asn Val Leu Tyr Gly Phe Pro Lys
        35                  40                  45

Phe Tyr Asp Ala Cys Ile Ala Asn Asn Ile Lys Pro Ile Phe Gly Met
    50                  55                  60

Thr Ile Tyr Val Thr Asn Gly Leu Asn Thr Val Glu Thr Val Val Leu
65                  70                  75                  80

Ala Lys Asn Asn Asp Gly Leu Lys Asp Leu Tyr Gln Leu Ser Ser Glu
                85                  90                  95

Ile Lys Met Asn Ala Leu Glu His Val Ser Phe Glu Leu Leu Lys Arg
            100                 105                 110

Phe Ser Asn Asn Met Ile Ile Ile Phe Lys Lys Val Gly Asp Gln His
        115                 120                 125

Arg Asp Ile Val Gln Val Phe Glu Thr His Asn Asp Thr Tyr Met Asp
    130                 135                 140

His Leu Ser Ile Ser Ile Gln Gly Arg Lys His Val Trp Ile Gln Asn
145                 150                 155                 160
```

```
Val Cys Tyr Gln Thr Arg Gln Asp Ala Asp Thr Ile Ser Ala Leu Ala
                165                 170                 175

Ala Ile Arg Asp Asn Thr Lys Leu Asp Leu Ile His Asp Gln Glu Asp
            180                 185                 190

Phe Gly Ala His Phe Leu Thr Glu Lys Glu Ile Asn Gln Leu Asp Ile
        195                 200                 205

Asn Gln Glu Tyr Leu Thr Gln Val Asp Val Ile Ala Gln Lys Cys Asp
    210                 215                 220

Ala Glu Leu Lys Tyr His Gln Ser Leu Leu Pro Gln Tyr Glu Thr Pro
225                 230                 235                 240

Asn Asp Glu Ser Ala Lys Lys Tyr Leu Trp Arg Val Leu Val Thr Gln
                245                 250                 255

Leu Lys Lys Leu Glu Leu Asn Tyr Asp Val Tyr Leu Glu Arg Leu Lys
            260                 265                 270

Tyr Glu Tyr Lys Val Ile Thr Asn Met Gly Phe Glu Asp Tyr Phe Leu
        275                 280                 285

Ile Val Ser Asp Leu Ile His Tyr Ala Lys Thr Asn Asp Val Met Val
    290                 295                 300

Gly Pro Gly Arg Gly Ser Ser Ala Gly Ser Leu Val Ser Tyr Leu Leu
305                 310                 315                 320

Gly Ile Thr Thr Ile Asp Pro Ile Lys Phe Asn Leu Leu Phe Glu Arg
                325                 330                 335

Phe Leu Asn Pro Glu Arg Val Thr Met Pro Asp Ile Asp Ile Asp Phe
            340                 345                 350

Glu Asp Thr Arg Arg Glu Arg Val Ile Gln Tyr Val Gln Glu Lys Tyr
        355                 360                 365

Gly Glu Leu His Val Ser Gly Ile Val Thr Phe Gly His Leu Leu Ala
    370                 375                 380

Arg Ala Val Ala Arg Asp Val Gly Arg Ile Met Gly Phe Asp Glu Val
385                 390                 395                 400

Thr Leu Asn Glu Ile Ser Ser Leu Ile Pro His Lys Leu Gly Ile Thr
                405                 410                 415

Leu Asp Glu Ala Tyr Gln Ile Asp Asp Phe Lys Glu Phe Val His Arg
            420                 425                 430

Asn His Arg His Glu Arg Trp Phe Ser Ile Cys Lys Lys Leu Glu Gly
        435                 440                 445

Leu Pro Arg His Thr Ser Thr His Ala Ala Gly Ile Ile Ile Asn Asp
    450                 455                 460

His Pro Leu Tyr Glu Tyr Ala Pro Leu Thr Lys Gly Asp Thr Gly Leu
465                 470                 475                 480

Leu Thr Gln Trp Thr Met Thr Glu Ala Glu Arg Ile Gly Leu Leu Lys
                485                 490                 495
```

Ile Asp Phe Leu Gly Leu Arg Asn Leu Ser Ile Ile His Gln Ile Leu
500 505 510

Thr Gln Val Lys Lys Asp Leu Gly Ile Asn Ile Asp Ile Glu Lys Ile
515 520 525

Pro Phe Asp Asp Gln Lys Val Phe Glu Leu Leu Ser Gln Gly Asp Thr
530 535 540

Thr Gly Ile Phe Gln Leu Glu Ser Asp Gly Val Arg Ser Val Leu Lys
545 550 555 560

Lys Leu Lys Pro Glu His Phe Glu Asp Ile Val Ala Val Thr Ser Leu
565 570 575

Tyr Arg Pro Gly Pro Met Glu Glu Ile Pro Thr Tyr Ile Thr Arg Arg
580 585 590

His Asp Pro Ser Lys Val Gln Tyr Leu His Pro His Leu Glu Pro Ile
595 600 605

Leu Lys Asn Thr Tyr Gly Val Ile Ile Tyr Gln Glu Gln Ile Met Gln
610 615 620

Ile Ala Ser Thr Phe Ala Asn Phe Ser Tyr Gly Glu Ala Asp Ile Leu
625 630 635 640

Arg Arg Ala Met Ser Lys Lys Asn Arg Ala Val Leu Glu Ser Glu Arg
645 650 655

Gln His Phe Ile Glu Gly Ala Lys Gln Asn Gly Tyr His Glu Asp Ile
660 665 670

Ser Lys Gln Ile Phe Asp Leu Ile Leu Lys Phe Ala Asp Tyr Gly Phe
675 680 685

Pro Arg Ala His Ala Val Ser Tyr Ser Lys Ile Ala Tyr Ile Met Ser
690 695 700

Phe Leu Lys Val His Tyr Pro Asn Tyr Phe Tyr Ala Asn Ile Leu Ser
705 710 715 720

Asn Val Ile Gly Ser Glu Lys Lys Thr Ala Gln Met Ile Glu Glu Ala
725 730 735

Lys Lys Gln Gly Ile Thr Ile Leu Pro Pro Asn Ile Asn Glu Ser His
740 745 750

Trp Phe Tyr Lys Pro Ser Gln Glu Gly Ile Tyr Leu Ser Ile Gly Thr
755 760 765

Ile Lys Gly Val Gly Tyr Gln Ser Val Lys Val Ile Val Asp Glu Arg
770 775 780

Tyr Gln Asn Gly Lys Phe Lys Asp Phe Phe Asp Phe Ala Arg Arg Ile
785 790 795 800

Pro Lys Arg Val Lys Thr Arg Lys Leu Leu Glu Ala Leu Ile Leu Val
805 810 815

Gly Ala Phe Asp Ala Phe Gly Lys Thr Arg Ser Thr Leu Leu Gln Ala
820 825 830

```
Ile Asp Gln Val Leu Asp Gly Asp Leu Asn Ile Glu Gln Asp Gly Phe
        835                 840                 845

Leu Phe Asp Ile Leu Thr Pro Lys Gln Met Tyr Glu Asp Lys Glu Glu
    850                 855                 860

Leu Pro Asp Ala Leu Ile Ser Gln Tyr Glu Lys Glu Tyr Leu Gly Phe
865                 870                 875                 880

Tyr Val Ser Gln His Pro Val Asp Lys Lys Phe Val Ala Lys Gln Tyr
                885                 890                 895

Leu Thr Ile Phe Lys Leu Ser Asn Ala Gln Asn Tyr Lys Pro Ile Leu
            900                 905                 910

Val Gln Phe Asp Lys Val Lys Gln Ile Arg Thr Lys Asn Gly Gln Asn
        915                 920                 925

Met Ala Phe Val Thr Leu Asn Asp Gly Ile Glu Thr Leu Asp Gly Val
    930                 935                 940

Ile Phe Pro Asn Gln Phe Lys Lys Tyr Glu Glu Leu Leu Ser His Asn
945                 950                 955                 960

Asp Leu Phe Ile Val Ser Gly Lys Phe Asp His Arg Lys Gln Gln Arg
                965                 970                 975

Gln Leu Ile Ile Asn Glu Ile Gln Thr Leu Ala Thr Phe Glu Glu Gln
            980                 985                 990

Lys Leu Ala Phe Ala Lys Gln Ile Ile Ile Arg Asn Lys Ser Gln Ile
        995                1000                1005

Asp Met Phe Glu Glu Met Ile Lys Ala Thr Lys Glu Asn Ala Asn Asp
   1010                1015                1020

Val Val Leu Ser Phe Tyr Asp Glu Thr Ile Lys Gln Met Thr Thr Leu
1025                1030                1035                1040

Gly Tyr Ile Asn Gln Lys Asp Ser Met Phe Asn Asn Phe Ile Gln Ser
                1045                1050                1055

Phe Asn Pro Ser Asp Ile Arg Leu Ile
            1060                1065
```

The present invention also relates to the *S. aureus dnaX* gene. This *S. aureus dnaX* gene has a partial nucleotide sequence corresponding to SEQ. ID. No. 3 as follows:

```
ttgaattatc aagccttata tcgtatgtac agaccccaaa gtttcgagga tgtcgtcgga      60
caagaacatg tcacgaagac attgcgcaat gcgatttcga aagaaaaaca gtcgcatgca     120
tatattttta gtggtccgag aggtacgggg aaaacgagta ttgccaaagt gtttgctaaa     180
gcaatcaact gtttaaatag cactgatgga gaaccttgta atgaatgtca tatttgtaaa     240
ggcattacgc aggggactaa ttcagatgtg atagaaattg atgctgctag taataatggc     300
gttgatgaaa taagaaatat tagagacaaa gttaaatatg caccaagtga atcgaaatat     360
aaagtttata ttatagatga ggtgcacatg ctaacaacag gtgcttttaa tgccctttta     420
aagacgttag aagaacctcc agcacacgct atttttatat tggcaacgac agaaccacat     480
aaaatccctc caacaatcat ttctagggca caacgttttg atttaaagc aattagccta      540
gatcaaattg ttgaacgttt aaaatttgta gcagatgcac aacaaattga atgtgaagat     600
gaagccttgg catttatcgc taaagcgtct gaaggggtga tgcgtgatgc attaagtatt     660
```

```
atggatcagg ctattgcttt cggcgatggc acattgacat tacaagatgc cctaaatgtt     720
acgggtagcg ttcatgatga agcgttggat cacttgtttg atgatattgt acaaggtgac     780
gtacaagcat cttttaaaaa ataccatcag tttataacag aaggtaaaga agtgaatcgc     840
ctaataaatg atatgattta ttttgtcaga gatacgatta tgaataaaac atctgagaaa     900
gatactgagt atcgagcact gatgaactta gaattagata tgttatatca aatgattgat     960
cttattaatg atacattagt gtcgattcgt tttagtgtga atcaaaacgt tcattttgaa    1020
gtattgttag taaaattagc tgagcagatt aagggtcaac cacaagtgat tgcgaatgta    1080
gctgaaccag cacaaattgc ttcatcgcca aacacagatg tattgttgca acgtatggaa    1140
cagttagagc aagaactaaa aacactaaaa gcacaaggag tgagtgttgc tcctactcaa    1200
aaatcttcga aaaagcctgc gagaggtata caaaaatcta aaaatgcatt ttcaatgcaa    1260
caaattgcaa aagtgctaga taaagcgaat aaggcagata tcaaattgtt gaaagatcat    1320
tggcaagaag tgattgacca tgcccaaaac aatgataaaa aatcactcgt tagtttattg    1380
caaaattcgg aacctgtggc ggcaagtgaa gatcacgtcc ttgtgaaatt tgaggaagag    1440
atccattgtg aaatcgtcaa taaagacgac gagaaacgta gtagtataga aagtgttgta    1500
tgtaatatcg ttaataaaaa cgttaaagtt gttggtgtac catcagatca atggcaaaga    1560
gttcgaacgg agtatttaca aaatcgtaaa aacgaaggcg atgatatgcc aaagcaacaa    1620
gcacaacaaa cagatattgc tcaaaaagca aaagatcttt tcggtgaaga aactgtacat    1680
gtgatagatg aagagtga                                                  1698
```

The *S. aureus dnaX* encoded protein (i.e., the tau subunit) has a partial amino acid sequence corresponding to SEQ. ID. No. 4 as follows:

```
Leu Asn Tyr Gln Ala Leu Tyr Arg Met Tyr Arg Pro Gln Ser Phe Glu
1               5                  10                  15

Asp Val Val Gly Gln Glu His Val Thr Lys Thr Leu Arg Asn Ala Ile
            20                  25                  30

Ser Lys Glu Lys Gln Ser His Ala Tyr Ile Phe Ser Gly Pro Arg Gly
        35                  40                  45

Thr Gly Lys Thr Ser Ile Ala Lys Val Phe Ala Lys Ala Ile Asn Cys
    50                  55                  60

Leu Asn Ser Thr Asp Gly Glu Pro Cys Asn Glu Cys His Ile Cys Lys
65                  70                  75                  80

Gly Ile Thr Gln Gly Thr Asn Ser Asp Val Ile Glu Ile Asp Ala Ala
                85                  90                  95

Ser Asn Asn Gly Val Asp Glu Ile Arg Asn Ile Arg Asp Lys Val Lys
            100                 105                 110

Tyr Ala Pro Ser Glu Ser Lys Tyr Lys Val Tyr Ile Ile Asp Glu Val
        115                 120                 125

His Met Leu Thr Thr Gly Ala Phe Asn Ala Leu Leu Lys Thr Leu Glu
    130                 135                 140

Glu Pro Pro Ala His Ala Ile Phe Ile Leu Ala Thr Thr Glu Pro His
145                 150                 155                 160

Lys Ile Pro Pro Thr Ile Ile Ser Arg Ala Gln Arg Phe Asp Phe Lys
                165                 170                 175

Ala Ile Ser Leu Asp Gln Ile Val Glu Arg Leu Lys Phe Val Ala Asp
            180                 185                 190

Ala Gln Gln Ile Glu Cys Glu Asp Glu Ala Leu Ala Phe Ile Ala Lys
        195                 200                 205
```

```
Ala Ser Glu Gly Gly Met Arg Asp Ala Leu Ser Ile Met Asp Gln Ala
    210                 215                 220

Ile Ala Phe Gly Asp Gly Thr Leu Thr Leu Gln Asp Ala Leu Asn Val
225                 230                 235                 240

Thr Gly Ser Val His Asp Glu Ala Leu Asp His Leu Phe Asp Asp Ile
                245                 250                 255

Val Gln Gly Asp Val Gln Ala Ser Phe Lys Lys Tyr His Gln Phe Ile
            260                 265                 270

Thr Glu Gly Lys Glu Val Asn Arg Leu Ile Asn Asp Met Ile Tyr Phe
        275                 280                 285

Val Arg Asp Thr Ile Met Asn Lys Thr Ser Glu Lys Asp Thr Glu Tyr
    290                 295                 300

Arg Ala Leu Met Asn Leu Glu Leu Asp Met Leu Tyr Gln Met Ile Asp
305                 310                 315                 320

Leu Ile Asn Asp Thr Leu Val Ser Ile Arg Phe Ser Val Asn Gln Asn
                325                 330                 335

Val His Phe Glu Val Leu Leu Val Lys Leu Ala Glu Gln Ile Lys Gly
            340                 345                 350

Gln Pro Gln Val Ile Ala Asn Val Ala Glu Pro Ala Gln Ile Ala Ser
        355                 360                 365

Ser Pro Asn Thr Asp Val Leu Leu Gln Arg Met Glu Gln Leu Glu Gln
    370                 375                 380

Glu Leu Lys Thr Leu Lys Ala Gln Gly Val Ser Val Ala Pro Thr Gln
385                 390                 395                 400

Lys Ser Ser Lys Lys Pro Ala Arg Gly Ile Gln Lys Ser Lys Asn Ala
                405                 410                 415

Phe Ser Met Gln Gln Ile Ala Lys Val Leu Asp Lys Ala Asn Lys Ala
            420                 425                 430

Asp Ile Lys Leu Leu Lys Asp His Trp Gln Glu Val Ile Asp His Ala
        435                 440                 445

Gln Asn Asn Asp Lys Lys Ser Leu Val Ser Leu Leu Gln Asn Ser Glu
    450                 455                 460

Pro Val Ala Ala Ser Glu Asp His Val Leu Val Lys Phe Glu Glu Glu
465                 470                 475                 480

Ile His Cys Glu Ile Val Asn Lys Asp Asp Glu Lys Arg Ser Ser Ile
                485                 490                 495

Glu Ser Val Val Cys Asn Ile Val Asn Lys Asn Val Lys Val Val Gly
            500                 505                 510

Val Pro Ser Asp Gln Trp Gln Arg Val Arg Thr Glu Tyr Leu Gln Asn
        515                 520                 525

Arg Lys Asn Glu Gly Asp Asp Met Pro Lys Gln Gln Ala Gln Gln Thr
    530                 535                 540
```

```
Asp Ile Ala Gln Lys Ala Lys Asp Leu Phe Gly Glu Glu Thr Val His
545                 550                 555                 560

Val Ile Asp Glu Glu Glx
                565
```

The tau subunit of *S. aureus* functions as does both the tau subunit and the gamma subunit of *E. coli*.

This invention also relates to the partial nucleotide sequence of the *S. aureus dnaB* gene. The partial nucleotide sequence of this *dnaB* gene corresponds to SEQ. ID. No. 5 as follows:

```
atggatagaa tgtatgagca aaatcaaatg ccgcataaca atgaagctga acagtctgtc 60
ttaggttcaa ttattataga tccagaattg attaatacta ctcaggaagt tttgcttcct 120
gagtcgtttt ataggggtgc ccatcaacat attttccgtg caatgatgca cttaaatgaa 180
gataataaag aaattgatgt tgtaacattg atggatcaat tatcgacgga aggtacgttg 240
aatgaagcgg gtggcccgca atatcttgca gagttatcta caaatgtacc aacgacgcga 300
aatgttcagt attatactga tatcgtttct aagcatgcat taaaacgtag attgattcaa 360
actgcagata gtattgccaa tgatggatat aatgatgaac ttgaactaga tgcgatttta 420
agtgatgcag aacgtcgaat tttagagcta tcatcttctc gtgaaagcga tggctttaaa 480
gacattcgag acgtcttagg acaagtgtat gaaacagctg aagagcttga tcaaaatagt 540
ggtcaaacac caggtatacc tacaggatat cgagatttag accaaatgac agcagggttc 600
aaccgaaatg atttaattat ccttgcagcg cgtccatctg taggtaagac tgcgttcgca 660
cttaatattg cacaaaaagt tgcaacgcat gaagatatgt atacagttgg tattttctcg 720
ctagagatgg gtgctgatca gttagccaca cgtatgattt gtagttctgg aaatgttgac 780
tcaaaccgct taagaacggg tactatgact gaggaagatt ggagtcgttt tactatagcg 840
gtaggtaaat tatcacgtac gaagattttt attgatgata caccgggtat tcgaattaat 900
gatttacgtt ctaaatgtcg tcgattaaag caagaacatg gcttagacat gattgtgatt 960
gactacttac agttgattca aggtagtggt tcacgtgcgt ccgataacag acaacaggaa 1020
gtttctgaaa tctctcgtac attaaaagca ttagcccgtg aattaaaatg tccagttatc 1080
gcattaagtc agttatctcg tggtgttgaa caacgacaag ataaacgtcc aatgatgagt 1140
gatattcgtg aatctggttc gattgagcaa gatgccgata tcgttgcatt cttataccgt 1200
gatgattact ataaccgtgg cggcgatgaa gatgatgacg atgatggtgg tttcgagcca 1260
caaacgaatg atgaaaacgg tgaaattgaa attatcattg ctaagcaacg taacggtcca 1320
acaggcacag ttaagttaca ttttatgaaa caatataata aatttaccga tatcgattat 1380
gcacatgcag atatgatg                                              1398
```

The amino acid sequence of *S. aureus* DnaB encoded by the *dnaB* gene corresponds to SEQ. ID. No. 6 as follows:

```
Met Asp Arg Met Tyr Glu Gln Asn Gln Met Pro His Asn Asn Glu Ala
  1               5                  10                  15

Glu Gln Ser Val Leu Gly Ser Ile Ile Ile Asp Pro Glu Leu Ile Asn
             20                  25                  30

Thr Thr Gln Glu Val Leu Leu Pro Glu Ser Phe Tyr Arg Gly Ala His
         35                  40                  45

Gln His Ile Phe Arg Ala Met Met His Leu Asn Glu Asp Asn Lys Glu
     50                  55                  60

Ile Asp Val Val Thr Leu Met Asp Gln Leu Ser Thr Glu Gly Thr Leu
 65                  70                  75                  80
```

```
Asn Glu Ala Gly Gly Pro Gln Tyr Leu Ala Glu Leu Ser Thr Asn Val
                  85                  90                  95

Pro Thr Thr Arg Asn Val Gln Tyr Tyr Thr Asp Ile Val Ser Lys His
             100                 105                 110

Ala Leu Lys Arg Arg Leu Ile Gln Thr Ala Asp Ser Ile Ala Asn Asp
         115                 120                 125

Gly Tyr Asn Asp Glu Leu Glu Leu Asp Ala Ile Leu Ser Asp Ala Glu
     130                 135                 140

Arg Arg Ile Leu Glu Leu Ser Ser Ser Arg Glu Ser Asp Gly Phe Lys
145                 150                 155                 160

Asp Ile Arg Asp Val Leu Gly Gln Val Tyr Glu Thr Ala Glu Glu Leu
                 165                 170                 175

Asp Gln Asn Ser Gly Gln Thr Pro Gly Ile Pro Thr Gly Tyr Arg Asp
             180                 185                 190

Leu Asp Gln Met Thr Ala Gly Phe Asn Arg Asn Asp Leu Ile Ile Leu
         195                 200                 205

Ala Ala Arg Pro Ser Val Gly Lys Thr Ala Phe Ala Leu Asn Ile Ala
     210                 215                 220

Gln Lys Val Ala Thr His Glu Asp Met Tyr Thr Val Gly Ile Phe Ser
225                 230                 235                 240

Leu Glu Met Gly Ala Asp Gln Leu Ala Thr Arg Met Ile Cys Ser Ser
                 245                 250                 255

Gly Asn Val Asp Ser Asn Arg Leu Arg Thr Gly Thr Met Thr Glu Glu
             260                 265                 270

Asp Trp Ser Arg Phe Thr Ile Ala Val Gly Lys Leu Ser Arg Thr Lys
         275                 280                 285

Ile Phe Ile Asp Asp Thr Pro Gly Ile Arg Ile Asn Asp Leu Arg Ser
     290                 295                 300

Lys Cys Arg Arg Leu Lys Gln Glu His Gly Leu Asp Met Ile Val Ile
305                 310                 315                 320

Asp Tyr Leu Gln Leu Ile Gln Gly Ser Gly Ser Arg Ala Ser Asp Asn
                 325                 330                 335

Arg Gln Gln Glu Val Ser Glu Ile Ser Arg Thr Leu Lys Ala Leu Ala
             340                 345                 350

Arg Glu Leu Lys Cys Pro Val Ile Ala Leu Ser Gln Leu Ser Arg Gly
         355                 360                 365

Val Glu Gln Arg Gln Asp Lys Arg Pro Met Met Ser Asp Ile Arg Glu
     370                 375                 380

Ser Gly Ser Ile Glu Gln Asp Ala Asp Ile Val Ala Phe Leu Tyr Arg
385                 390                 395                 400

Asp Asp Tyr Tyr Asn Arg Gly Gly Asp Glu Asp Asp Asp Asp Asp Gly
                 405                 410                 415
```

```
Gly Phe Glu Pro Gln Thr Asn Asp Glu Asn Gly Glu Ile Glu Ile Ile
            420                 425                 430

Ile Ala Lys Gln Arg Asn Gly Pro Thr Gly Thr Val Lys Leu His Phe
        435                 440                 445

Met Lys Gln Tyr Asn Lys Phe Thr Asp Ile Asp Tyr Ala His Ala Asp
    450                 455                 460

Met Met
465
```

The present invention also relates to the *S. aureus polC* gene (encoding Pol III-L or α-large). The partial nucleotide sequence of this *polC* gene corresponds to SEQ. ID. No. 7 as follows:

```
atgacagagc aacaaaaatt taaagtgctt gctgatcaaa ttaaaatttc aaatcaatta 60
gatgctgaaa ttttaaattc aggtgaactg acacgtatag atgtttctaa caaaaacaga 120
acatgggaat ttcatattac attaccacaa ttcttagctc atgaagatta tttattattt 180
ataaatgcaa tagagcaaga gtttaaagat atcgccaacg ttacatgtcg ttttacggta 240
acaaatggca cgaatcaaga tgaacatgca attaaatact ttgggcactg tattgaccaa 300
acagctttat ctccaaaagt taaaggtcaa ttgaaacaga aaaagcttat tatgtctgga 360
aaagtattaa aagtaatggt atcaaatgac attgaacgta atcattttga taaggcatgt 420
aatggaagtc ttatcaaagc gtttagaaat tgtggttttg atatcgataa aatcatattc 480
gaaacaaatg ataatgatca agaacaaaac ttagcttctt tagaagcaca tattcaagaa 540
gaagacgaac aaagtgcacg attggcaaca gagaaacttg aaaaaatgaa agctgaaaaa 600
gcgaaacaac aagataacaa cgaaagtgct gtcgataagt gtcaaattgg taagccgatt 660
caaattgaaa atattaaacc aattgaatct attattgagg aagagtttaa agttgcaata 720
gagggtgtca tttttgatat aaacttaaaa gaacttaaaa gtggtcgcca tatcgtagaa 780
attaaagtga ctgactatac ggactcttta gttttaaaaa tgtttactcg taaaaacaaa 840
gatgatttag aacattttaa agcgctaagt gttggtaaat gggttagggc tcaaggtcgt 900
attgaagaag atacatttat tagagattta gttatgatga tgtctgatat tgaagagatt 960
aaaaaagcga caaaaaaaga taaggctgaa gaaaagcgtg tagaattcca cttgcatact 1020
gcaatgagcc aaatggatgg tatacccaat attggtgcgt atgttaaaca ggcagcagac 1080
tggggacatc cagccattgc ggttacagac cataatgttg tgcaagcatt tccagatgct 1140
cacgcagcag cggaaaaaca tggcattaaa atgatatacg gtatggaagg tatgttagtt 1200
gatgatggtg ttccgattgc atacaaacca caagatgtcg tattaaaaga tgctacttat 1260
gttgtgttcg acgttgagac aactggttta tcaaatcagt atgataaaat catcgagctt 1320
gcagctgtga aagttcataa cggtgaaatc atcgataagt ttgaaaggtt tagtaatccg 1380
catgaacgat tatcggaaac gattatcaat ttgacgcata ttactgatga tatgttagta 1440
gatgcccctg agattgaaga agtacttaca gagtttaaag aatgggttgg cgatgcgata 1500
ttcgtagcgc ataatgcttc gtttgatatg ggcttcatcg atacgggata tgaacgtctt 1560
gggtttggac catcaacgaa tggtgttatc gatactttag aattatctcg tacgattaat 1620
actgaatatg gtaaacatgg tttgaatttc ttggctaaaa aatatggcgt agaattaacg 1680
caacatcacc gtgccattta tgatacagaa gcaacagctt acattttcat aaaaatggtt 1740
caacaaatga aagaattagg cgtattaaat cataacgaaa tcaacaaaaa actcagtaat 1800
gaagatgcat ataaacgtgc aagacctagt catgtcacat taattgtaca aaaccaacaa 1860
ggtcttaaaa atctatttaa aattgtaagt gcatcattgg tgaagtattt ctaccgtaca 1920
cctcgaattc cacgttcatt gttagatgaa tatcgtgagg gattattggt aggtacagcg 1980
tgtgatgaag gtgaattatt tacggcagtt atgcagaagg accagagtca agttgaaaaa 2040
attgccaaat attatgattt tattgaaatt caaccaccgg cactttatca agatttaatt 2100
gatagagagc ttattagaga tactgaaaca ttacatgaaa tttatcaacg tttaatacat 2160
gcaggtgaca cagcgggtat acctgttatt gcgacaggaa atgcacacta tttgtttgaa 2220
catgatggta tcgcacgtaa aattttaata gcatcacaac ccggcaatcc acttaatcgc 2280
tcaactttac cggaagcaca ttttagaact acagatgaaa tgttaaacga gtttcatttt 2340
ttaggtgaag aaaaagcgca tgaaattgtt gtgaaaaata caaacgaatt agcagatcga 2400
attgaacgtg ttgttcctat taaagatgaa ttatacacac cgcgtatgga aggtgctaac 2460
gaagaaatta gagaactaag ttatgcaaat gcgcgtaaac tgtatggtga agacctgcct 2520
caaatcgtaa ttgatcgatt agaaaaagaa ttaaaaagta ttatcggtaa tggatttgcg 2580
gtaatttact taatttcgca acgtttagtt aaaaaatcat tagatgatgg atacttagtt 2640
ggttcccgtg gttcagtagg ttctagtttt gtagcgacaa tgactgagat tactgaagta 2700
```

```
aacccgttac cgccacacta tatttgtccg aactgtaaaa cgagtgaatt tttcaatgat 2760
ggttcagtag gatcaggatt tgatttacct gataagacgt gtgaaacttg tggagcgcca 2820
cttattaaag aaggacaaga tattccgttt gaaacatttt taggatttaa gggagataaa 2880
gttcctgata tcgacttaaa ctttagtggt gaatatcaac cgaatgccca taactacaca 2940
aaagtattat ttggtgagga taaagtattc cgtgcaggta caattggtac tgttgctgaa 3000
aagactgctt ttggttatgt taaaggttat ttgaatgatc aaggtatcca caaaagaggt 3060
gctgaaatag atcgactcgt taaaggatgt acaggtgtta aacgtacaac tggacagcat 3120
ccaggggta ttattgtagt acctgattac atggatattt atgattttac gccgatacaa 3180
tatcctgccg atgatcaaaa ttcagcatgg atgacgacac attttgattt ccattctatt 3240
catgataatg tattaaaact tgatatactt ggacacgatg atccaacaat gattcgtatg 3300
cttcaagatt tatcaggaat tgatccaaaa acaatacctg tagatgataa agaagttatg 3360
cagatattta gtacacctga aagtttgggt gttactgaag atgaaatttt atgtaaaaca 3420
ggtacatttg gggtaccaga attcggtaca ggattcgtgc gtcaaatgtt agaagataca 3480
aagccaacaa cattttctga attagttcaa atctcaggat tatctcatgg tacagatgtg 3540
tggttaggca atgctcaaga attaattaaa accggtatat gtgatttatc aagtgtaatt 3600
ggttgtcgtg atgatatcat ggtttattta atgtatgctg gtttagaacc atcaatggct 3660
tttaaaataa tggagtcagt acgtaaaggt aaaggtttaa ctgaagaaat gattgaaacg 3720
atgaaagaaa atgaagtgcc agattggtat ttagattcat gtcttaaaat taagtacatg 3780
ttccctaaag cccatgcagc agcatacgtt ttaatggcag tacgtatcgc atatttcaaa 3840
gtacatcatc cactttatta ctatgcatct tactttacaa ttcgtgcgtc agactttgat 3900
ttaatcacga tgattaaaga taaaacaagc attcgaaata ctgtaaaaga catgtattct 3960
cgctatatgg atctaggtaa aaaagaaaaa gacgtattaa cagtcttgga aattatgaat 4020
gaaatggcgc atcgaggtta tcgaatgcaa ccgattagtt tagaaaagag tcaggcgttc 4080
gaatttatca ttgaaggcga tacacttatt ccgccgttca tatcagtgcc tgggcttggc 4140
gaaaacgttg cgaaacgaat tgttgaagct cgtgacgatg gcccattttt atcaaaagaa 4200
gatttaaaca aaaaagctgg attatctcag aaaattattg agtatttaga tgagttaggc 4260
tcattaccga atttaccaga taaagctcaa ctttcgatat ttgatatg              4308
```

The amino acid sequence of the *S. aureus polC* gene product, α-large, corresponds to SEQ. ID. No. 8 as follows:

```
Met Thr Glu Gln Gln Lys Phe Lys Val Leu Ala Asp Gln Ile Lys Ile
  1               5                  10                  15

Ser Asn Gln Leu Asp Ala Glu Ile Leu Asn Ser Gly Glu Leu Thr Arg
             20                  25                  30

Ile Asp Val Ser Asn Lys Asn Arg Thr Trp Glu Phe His Ile Thr Leu
         35                  40                  45

Pro Gln Phe Leu Ala His Glu Asp Tyr Leu Leu Phe Ile Asn Ala Ile
     50                  55                  60

Glu Gln Glu Phe Lys Asp Ile Ala Asn Val Thr Cys Arg Phe Thr Val
 65                  70                  75                  80

Thr Asn Gly Thr Asn Gln Asp Glu His Ala Ile Lys Tyr Phe Gly His
                 85                  90                  95

Cys Ile Asp Gln Thr Ala Leu Ser Pro Lys Val Lys Gly Gln Leu Lys
            100                 105                 110

Gln Lys Lys Leu Ile Met Ser Gly Lys Val Leu Lys Val Met Val Ser
        115                 120                 125

Asn Asp Ile Glu Arg Asn His Phe Asp Lys Ala Cys Asn Gly Ser Leu
    130                 135                 140

Ile Lys Ala Phe Arg Asn Cys Gly Phe Asp Ile Asp Lys Ile Ile Phe
145                 150                 155                 160
```

```
Glu Thr Asn Asp Asn Asp Gln Glu Gln Asn Leu Ala Ser Leu Glu Ala
                  165                 170                 175

His Ile Gln Glu Glu Asp Glu Gln Ser Ala Arg Leu Ala Thr Glu Lys
              180                 185                 190

Leu Glu Lys Met Lys Ala Glu Lys Ala Lys Gln Gln Asp Asn Lys Gln
          195                 200                 205

Ser Ala Val Asp Lys Cys Gln Ile Gly Lys Pro Ile Gln Ile Glu Asn
      210                 215                 220

Ile Lys Pro Ile Glu Ser Ile Ile Glu Glu Glu Phe Lys Val Ala Ile
225                 230                 235                 240

Glu Gly Val Ile Phe Asp Ile Asn Leu Lys Glu Leu Lys Ser Gly Arg
                  245                 250                 255

His Ile Val Glu Ile Lys Val Thr Asp Tyr Thr Asp Ser Leu Val Leu
              260                 265                 270

Lys Met Phe Thr Arg Lys Asn Lys Asp Asp Leu Glu His Phe Lys Ala
          275                 280                 285

Leu Ser Val Gly Lys Trp Val Arg Ala Gln Gly Arg Ile Glu Glu Asp
      290                 295                 300

Thr Phe Ile Arg Asp Leu Val Met Met Met Ser Asp Ile Glu Glu Ile
305                 310                 315                 320

Lys Lys Ala Thr Lys Lys Asp Lys Ala Glu Glu Lys Arg Val Glu Phe
                  325                 330                 335

His Leu His Thr Ala Met Ser Gln Met Asp Gly Ile Pro Asn Ile Gly
              340                 345                 350

Ala Tyr Val Lys Gln Ala Ala Asp Trp Gly His Pro Ala Ile Ala Val
          355                 360                 365

Thr Asp His Asn Val Val Gln Ala Phe Pro Asp Ala His Ala Ala Ala
      370                 375                 380

Glu Lys His Gly Ile Lys Met Ile Tyr Gly Met Glu Gly Met Leu Val
385                 390                 395                 400

Asp Asp Gly Val Pro Ile Ala Tyr Lys Pro Gln Asp Val Val Leu Lys
                  405                 410                 415

Asp Ala Thr Tyr Val Val Phe Asp Val Glu Thr Thr Gly Leu Ser Asn
              420                 425                 430

Gln Tyr Asp Lys Ile Ile Glu Leu Ala Ala Val Lys Val His Asn Gly
          435                 440                 445

Glu Ile Ile Asp Lys Phe Glu Arg Phe Ser Asn Pro His Glu Arg Leu
      450                 455                 460

Ser Glu Thr Ile Ile Asn Leu Thr His Ile Thr Asp Asp Met Leu Val
465                 470                 475                 480

Asp Ala Pro Glu Ile Glu Glu Val Leu Thr Glu Phe Lys Glu Trp Val
                  485                 490                 495
```

Gly Asp Ala Ile Phe Val Ala His Asn Ala Ser Phe Asp Met Gly Phe
500 505 510

Ile Asp Thr Gly Tyr Glu Arg Leu Gly Phe Gly Pro Ser Thr Asn Gly
515 520 525

Val Ile Asp Thr Leu Glu Leu Ser Arg Thr Ile Asn Thr Glu Tyr Gly
530 535 540

Lys His Gly Leu Asn Phe Leu Ala Lys Lys Tyr Gly Val Glu Leu Thr
545 550 555 560

Gln His His Arg Ala Ile Tyr Asp Thr Glu Ala Thr Ala Tyr Ile Phe
565 570 575

Ile Lys Met Val Gln Gln Met Lys Glu Leu Gly Val Leu Asn His Asn
580 585 590

Glu Ile Asn Lys Lys Leu Ser Asn Glu Asp Ala Tyr Lys Arg Ala Arg
595 600 605

Pro Ser His Val Thr Leu Ile Val Gln Asn Gln Gln Gly Leu Lys Asn
610 615 620

Leu Phe Lys Ile Val Ser Ala Ser Leu Val Lys Tyr Phe Tyr Arg Thr
625 630 635 640

Pro Arg Ile Pro Arg Ser Leu Leu Asp Glu Tyr Arg Glu Gly Leu Leu
645 650 655

Val Gly Thr Ala Cys Asp Glu Gly Glu Leu Phe Thr Ala Val Met Gln
660 665 670

Lys Asp Gln Ser Gln Val Glu Lys Ile Ala Lys Tyr Tyr Asp Phe Ile
675 680 685

Glu Ile Gln Pro Pro Ala Leu Tyr Gln Asp Leu Ile Asp Arg Glu Leu
690 695 700

Ile Arg Asp Thr Glu Thr Leu His Glu Ile Tyr Gln Arg Leu Ile His
705 710 715 720

Ala Gly Asp Thr Ala Gly Ile Pro Val Ile Ala Thr Gly Asn Ala His
725 730 735

Tyr Leu Phe Glu His Asp Gly Ile Ala Arg Lys Ile Leu Ile Ala Ser
740 745 750

Gln Pro Gly Asn Pro Leu Asn Arg Ser Thr Leu Pro Glu Ala His Phe
755 760 765

Arg Thr Thr Asp Glu Met Leu Asn Glu Phe His Phe Leu Gly Glu Glu
770 775 780

Lys Ala His Glu Ile Val Val Lys Asn Thr Asn Glu Leu Ala Asp Arg
785 790 795 800

Ile Glu Arg Val Val Pro Ile Lys Asp Glu Leu Tyr Thr Pro Arg Met
805 810 815

Glu Gly Ala Asn Glu Glu Ile Arg Glu Leu Ser Tyr Ala Asn Ala Arg
820 825 830

```
Lys Leu Tyr Gly Glu Asp Leu Pro Gln Ile Val Ile Asp Arg Leu Glu
        835                 840                 845

Lys Glu Leu Lys Ser Ile Ile Gly Asn Gly Phe Ala Val Ile Tyr Leu
    850                 855                 860

Ile Ser Gln Arg Leu Val Lys Lys Ser Leu Asp Asp Gly Tyr Leu Val
865                 870                 875                 880

Gly Ser Arg Gly Ser Val Gly Ser Ser Phe Val Ala Thr Met Thr Glu
                885                 890                 895

Ile Thr Glu Val Asn Pro Leu Pro Pro His Tyr Ile Cys Pro Asn Cys
            900                 905                 910

Lys Thr Ser Glu Phe Phe Asn Asp Gly Ser Val Gly Ser Gly Phe Asp
        915                 920                 925

Leu Pro Asp Lys Thr Cys Glu Thr Cys Gly Ala Pro Leu Ile Lys Glu
    930                 935                 940

Gly Gln Asp Ile Pro Phe Glu Lys Phe Leu Gly Phe Lys Gly Asp Lys
945                 950                 955                 960

Val Pro Asp Ile Asp Leu Asn Phe Ser Gly Glu Tyr Gln Pro Asn Ala
                965                 970                 975

His Asn Tyr Thr Lys Val Leu Phe Gly Glu Asp Lys Val Phe Arg Ala
            980                 985                 990

Gly Thr Ile Gly Thr Val Ala Glu Lys Thr Ala Phe Gly Tyr Val Lys
        995                1000                1005

Gly Tyr Leu Asn Asp Gln Gly Ile His Lys Arg Gly Ala Glu Ile Asp
   1010                1015                1020

Arg Leu Val Lys Gly Cys Thr Gly Val Lys Ala Thr Thr Gly Gln His
1025                1030                1035                1040

Pro Gly Gly Ile Ile Val Val Pro Asp Tyr Met Asp Ile Tyr Asp Phe
                1045                1050                1055

Thr Pro Ile Gln Tyr Pro Ala Asp Asp Gln Asn Ser Ala Trp Met Thr
            1060                1065                1070

Thr His Phe Asp Phe His Ser Ile His Asp Asn Val Leu Lys Leu Asp
        1075                1080                1085

Ile Leu Gly His Asp Asp Pro Thr Met Ile Arg Met Leu Gln Asp Leu
   1090                1095                1100

Ser Gly Ile Asp Pro Lys Thr Ile Pro Val Asp Asp Lys Glu Val Met
1105                1110                1115                1120

Gln Ile Phe Ser Thr Pro Glu Ser Leu Gly Val Thr Glu Asp Glu Ile
                1125                1130                1135

Leu Cys Lys Thr Gly Thr Phe Gly Val Pro Asn Ser Asp Arg Ile Arg
            1140                1145                1150

Arg Gln Met Leu Glu Asp Thr Lys Pro Thr Thr Ph  S r Glu Leu Val
        1155                1160                1165
```

```
Gln Ile Ser Gly Leu Ser His Gly Thr Asp Val Trp Leu Gly Asn Ala
   1170                1175                1180

Gln Glu Leu Ile Lys Thr Gly Ile Cys Asp Leu Ser Ser Val Ile Gly
1185                1190                1195                1200

Cys Arg Asp Asp Ile Met Val Tyr Leu Met Tyr Ala Gly Leu Glu Pro
                1205                1210                1215

Ser Met Ala Phe Lys Ile Met Glu Ser Val Arg Lys Gly Lys Gly Leu
            1220                1225                1230

Thr Glu Glu Met Ile Glu Thr Met Lys Glu Asn Glu Val Pro Asp Trp
        1235                1240                1245

Tyr Leu Asp Ser Cys Leu Lys Ile Lys Tyr Ile Phe Pro Lys Ala His
   1250                1255                1260

Ala Ala Ala Tyr Val Leu Met Ala Val Arg Ile Ala Tyr Phe Lys Val
1265                1270                1275                1280

His His Pro Leu Tyr Tyr Tyr Ala Ser Tyr Phe Thr Ile Arg Ala Ser
                1285                1290                1295

Asp Phe Asp Leu Ile Thr Met Ile Lys Asp Lys Thr Ser Ile Arg Asn
            1300                1305                1310

Thr Val Lys Asp Met Tyr Ser Arg Tyr Met Asp Leu Gly Lys Lys Glu
        1315                1320                1325

Lys Asp Val Leu Thr Val Leu Glu Ile Met Asn Glu Met Ala His Arg
   1330                1335                1340

Gly Tyr Arg Met Gln Pro Ile Ser Leu Glu Lys Ser Gln Ala Phe Glu
1345                1350                1355                1360

Phe Ile Ile Glu Gly Asp Thr Leu Ile Pro Pro Phe Ile Ser Val Pro
                1365                1370                1375

Gly Leu Gly Glu Asn Val Ala Lys Arg Ile Val Glu Ala Arg Asp Asp
            1380                1385                1390

Gly Pro Phe Leu Ser Lys Glu Asp Leu Asn Lys Lys Ala Gly Leu Tyr
        1395                1400                1405

Gln Lys Ile Ile Glu Tyr Leu Asp Glu Leu Gly Ser Leu Pro Asn Leu
   1410                1415                1420

Pro Asp Lys Ala Gln Leu Ser Ile Phe Asp Met
1425                1430                1435
```

This invention also relates to the *S. aureus dnaN* gene encoding the beta subunit. The partial nucleotide sequence of this *dnaN* gene corresponds to SEQ. ID. No. 9 as follows:

```
atgatggaat tcactattaa aagagattat tttattacac aattaaatga cacattaaaa      60
gctatttcac caagaacaac attacctata ttaactggta tcaaaatcga tgcgaaagaa     120
catgaagtta tattaactgg ttcagactct gaaatttcaa tagaaatcac tattcctaaa     180
actgtagatg gcgaagatat tgtcaatatt tcagaaacag gctcagtagt acttcctgga     240
```

cgattctttg ttgatattat aaaaaaatta cctggtaaag atgttaaatt atctacaaat 300
gaacaattcc agacattaat tacatcaggt cattctgaat ttaatttgag tggcttagat 360
ccagatcaat atcctttatt acctcaagtt tctagagatg acgcaattca attgtcggta 420
aaagtactta aaaacgtgat tgcacaaacg aattttgcag tgtccacctc agaaacacgc 480
ccagtactaa ctggtgtgaa ctggcttata caagaaaatg aattaatatg cacagcgact 540
gattcacacc gcttggctgt aagaaagttg cagttagaag atgtttctga aaacaaaaat 600
gtcatcattc caggtaaggc tttagctgaa ttaaataaaa ttatgtctga caatgaagaa 660
gacattgata tcttctttgc ttcaaaccaa gttttattta aagttggaaa tgtgaacttt 720
atttctcgat tattagaagg acattatcct gatacaagag gttttattgg tgaaaactat 780

```
Ser His Ala Tyr Leu Phe Glu Gly Asp Asp Ala Gln Thr Met Lys Gln
            20                  25                  30

Val Ala Ile Asn Phe Ala Lys Leu Ile Leu Cys Gln Thr Asp Ser Gln
        35                  40                  45

Cys Glu Thr Lys Val Ser Thr Tyr Asn His Pro Asp Phe Met Tyr Ile
    50                  55                  60

Ser Thr Thr Glu Asn Ala Ile Lys Lys Glu Gln Val Glu Gln Leu Val
65                  70                  75                  80

Arg His Met Asn Gln Leu Pro Ile Glu Ser Thr Asn Lys Val Tyr Ile
                85                  90                  95

Ile Glu Asp Phe Glu Asp Phe Glu Lys Leu Thr Val Gln Gly Glu Asn
            100                 105                 110

Ser Ile Leu Lys Phe Leu Glu Glu Pro Pro Asp Asn Thr Ile Ala Ile
        115                 120                 125

Leu Leu Ser Thr Lys Pro Glu Gln Ile Leu Asp Thr Ile His Ser Arg
    130                 135                 140

Cys Gln His Val Tyr Phe Lys Pro Ile Asp Lys Glu Lys Phe Ile Asn
145                 150                 155                 160

Arg Leu Val Glu Gln Asn Met Ser Lys Pro Val Ala Glu Met Ile Ser
                165                 170                 175

Thr Tyr Thr Thr Gln Ile Asp Asn Ala Met Ala Leu Asn Glu Glu Phe
            180                 185                 190

Asp Leu Leu Ala Leu Arg Lys Ser Val Ile Arg Trp Glu Leu Leu Leu
        195                 200                 205

Thr Asn Lys Pro Met Ala Leu Ile Gly Ile Ile Asp Leu Leu Lys Gln
    210                 215                 220

Ala Lys Asn Lys Lys Leu Gln Ser Leu Thr Ile Ala Ala Val Asn Gly
225                 230                 235                 240

Phe Phe Glu Asp Ile Ile His Thr Lys Val Asn Val Glu Asp Lys Gln
                245                 250                 255

Ile Tyr Ser Asp Leu Lys Asn Asp Ile Asp Gln Tyr Ala Gln Lys Leu
            260                 265                 270

Ser Phe Asn Gln Leu Ile Leu Met Phe Asp Gln Leu Thr Glu Ala His
        275                 280                 285

Lys Lys Leu Asn Gln Asn Val Asn Pro Thr Leu Val Phe Glu Gln Ile
    290                 295                 300

Val Ile Lys Gly Val Ser
305                 310
```

This invention also relates to the *S. aureus holB* gene encoding the delta prime subunit. The partial nucleotide sequence of this *holB* gene corresponds to SEQ. ID. No. 13 as follows:

```
atgagcgaca atattgtagc tatttatgga gatgtgcctg aattggttga aaaacaaagt 60
gcagaaatca tatcacaatt tttgaaaagt gatagagatg actttaactt tgtgaaatat 120
aatttatacg aaacagagat tgcaccaatt gttgaagaaa cattaacatt gcctttcttt 180
tcagataaaa aagcaatttt ggttaaaaat gcatatatat ttacaggtga aaaagcgcca 240
aaagatatgg ctcataatgt agaccaatta atagaattta ttgaaaaata tgatggcgaa 300
aatttgattg tctttgagat atatcaaaat aaacttgatg aaagaaaaaa gttaactaaa 360
actctaaaaa agcatgcaag gcttaaaaaa atagaagcaga tgtcggaggg gatcaagtgg 420
```

Glu Ala Thr Gln S r Asn Ser Asn Val Gln Ile Ala Ser Asp Asp Leu
65 70 75 80

Gln Met Ile Glu Met His Glu Leu Ile Gln Glu Phe Tyr Tyr Tyr Ala
85 90 95

Leu Thr Lys Thr Val Glu Gly Glu Gln Ala Leu Thr Tyr Leu Gln Glu
100 105 110

Arg Gly Phe Thr Asp Ala Leu Ile Lys Glu Arg Gly Ile Gly Phe Ala
115 120 125

Pro Asp Ser Ser His Phe Cys His Asp Phe Leu Gln Lys Lys Gly Tyr
130 135 140

Asp Ile Glu Leu Ala Tyr Glu Ala Gly Leu Leu Ser Arg Asn Glu Glu
145 150 155 160

Asn Phe Ser Tyr Tyr Asp Arg Phe Arg Asn Arg Ile Met Phe Pro Leu
165 170 175

Lys Asn Ala Gln Gly Arg Ile Val Gly Tyr Ser Gly Arg Thr Tyr Thr
180 185 190

Gly Gln Glu Pro Lys Tyr Leu Asn Ser Pro Glu Thr Pro Ile Phe Gln
195 200 205

Lys Arg Lys Leu Leu Tyr Asn Leu Asp Lys Ala Arg Lys Ser Ile Arg
210 215 220

Lys Leu Asp Glu Ile Val Leu Leu Glu Gly Phe Met Asp Val Ile Lys
225 230 235 240

Ser Asp Thr Ala Gly Leu Lys Asn Val Val Ala Thr Met Gly Thr Gln
245 250 255

Leu Ser Asp Glu His Ile Thr Phe Ile Arg Lys Leu Thr Ser Asn Ile
260 265 270

Thr Leu Met Phe Asp Gly Asp Phe Ala Gly Ser Glu Ala Thr Leu Lys
275 280 285

Thr Gly Gln His Leu Leu Gln Gln Gly Leu Asn Val Phe Val Ile Gln
290 295 300

Leu Pro Ser Gly Met Asp Pro Asp Glu Tyr Ile Gly Lys Tyr Gly Asn
305 310 315 320

Asp Ala Phe Thr Thr Phe Val Lys Asn Asp Lys Lys Ser Phe Ala His
325 330 335

Tyr Lys Val Ser Ile Leu Lys Asp Glu Ile Ala His Asn Asp Leu Ser
340 345 350

Tyr Glu Arg Tyr Leu Lys Glu Leu Ser His Asp Ile Ser Leu Met Lys
355 360 365

Ser Ser Ile Leu Gln Gln Lys Ala Ile Asn Asp Val Ala Pro Phe Phe
370 375 380

Asn Val Ser Pro Glu Gln Leu Ala Asn Glu Ile Gln Phe Asn Gln Ala
385 390 395 400

Pro Ala Asn Tyr Tyr Pro Glu Asp Glu Tyr Gly Gly Tyr Asp Glu Tyr
405 410 415

Gly Gly Tyr Ile Glu Pro Glu Pro Ile Gly Met Ala Gln Phe Asp Asn
420 425 430

Leu Ser Arg Arg Glu Lys Ala Glu Arg Ala Phe Leu Lys His Leu Met
435 440 445

```
Lys Val His Ser Val Ser Arg Leu Trp Glu Phe His Phe Ala Phe Ala
        35                  40                  45

Ala Val Leu Pro Ile Ala Thr Tyr Arg Glu Leu His Asp Arg Leu Ile
    50                  55                  60

Arg Thr Phe Glu Ala Ala Asp Ile Lys Val Thr Phe Asp Ile Gln Ala
65                  70                  75                  80

Ala Gln Val Asp Tyr Ser Asp Asp Leu Leu Gln Ala Tyr Tyr Gln Glu
                85                  90                  95

Ala Phe Glu His Ala Pro Cys Asn Ser Ala Ser Phe Lys Ser Ser Phe
            100                 105                 110

Ser Lys Leu Lys Val Thr Tyr Glu Asp Asp Lys Leu Ile Ile Ala Ala
        115                 120                 125

Pro Gly Phe Val Asn Asn Asp His Phe Arg Asn Asn His Leu Pro Asn
    130                 135                 140

Leu Val Lys Gln Leu Glu Ala Phe Gly Phe Gly Ile Leu Thr Ile Asp
145                 150                 155                 160

Met Val Ser Asp Gln Glu Met Thr Glu His Leu Thr Lys Asn Phe Val
                165                 170                 175

Ser Ser Arg Gln Ala Leu Val Lys Lys Ala Val Gln Asp Asn Leu Glu
            180                 185                 190

Ala Gln Lys Ser Leu Glu Ala Met Met Pro Pro Val Glu Glu Ala Thr
        195                 200                 205

Pro Ala Pro Lys Phe Asp Tyr Lys Glu Arg Ala Ala Lys Arg Gln Ala
    210                 215                 220

Gly Phe Glu Lys Ala Thr Ile Thr Pro Met Ile Glu Ile Glu Thr Glu
225                 230                 235                 240

Glu Asn Arg Ile Val Phe Glu Gly Met Val Phe Asp Val Glu Arg Lys
                245                 250                 255

Thr Thr Arg Thr Gly Arg His Ile Ile Asn Phe Lys Met Thr Asp Tyr
            260                 265                 270

Thr Ser Ser Phe Ala Leu Gln Lys Trp Ala Lys Asp Asp Glu Glu Leu
        275                 280                 285

Arg Lys Phe Asp Met Ile Ala Lys Gly Ala Trp Leu Arg Val Gln Gly
    290                 295                 300

Asn Ile Glu Thr Asn Pro Phe Thr Lys Ser Leu Thr Met Asn Val Gln
305                 310                 315                 320

Gln Val Lys Glu Ile Val Arg His Glu Arg Lys Asp Leu Met Pro Glu
                325                 330                 335

Gly Gln Lys Arg Val Glu Leu His Ala His Thr Asn Met Ser Thr Met
            340                 345                 350

Asp Ala Leu Pro Thr Val Glu Ser Leu Il  Asp Thr Ala Ala Lys Trp
        355                 360                 365
```

```
Gly His Lys Ala Ile Ala Ile Thr Asp His Ala Asn Val Gln Ser Phe
    370                 375                 380

Pro His Gly Tyr His Arg Ala Arg Lys Ala Gly Ile Lys Ala Ile Phe
385                 390                 395                 400

Gly Leu Glu Ala Asn Ile Val Glu Asp Lys Val Pro Ile Ser Tyr Glu
                405                 410                 415
```

```
Gln Pro Leu Val Val Arg Glu Leu Ile Lys Asp Gln Ala Gly Ile Glu
705                 710                 715                 720

Gln Val Ile Arg Asp L u Ile Glu Val Gly Lys Arg Ala Lys Lys Pro
                725                 730                 735

Val Leu Ala Thr Gly Asn Val His Tyr Leu Glu Pro Glu Glu Glu Ile
            740                 745                 750

Tyr Arg Glu Ile Ile Val Arg Ser Leu Gly Gln Gly Ala Met Ile Asn
        755                 760                 765

Arg Thr Ile Gly Arg Gly Glu Gly Ala Gln Pro Ala Pro Leu Pro Lys
    770                 775                 780

Ala His Phe Arg Thr Thr Asn Glu Met Leu Asp Glu Phe Ala Phe Leu
785                 790                 795                 800

Gly Lys Asp Leu Ala Tyr Gln Val Val Val Gln Asn Thr Gln Asp Phe
                805                 810                 815

Ala Asp Arg Ile Glu Glu Val Glu Val Val Lys Gly Asp Leu Tyr Thr
            820                 825                 830

Pro Tyr Ile Asp Lys Ala Glu Glu Thr Val Ala Glu Leu Thr Tyr Gln
        835                 840                 845

Lys Ala Phe Glu Ile Tyr Gly Asn Pro Leu Pro Asp Ile Ile Asp Leu
    850                 855                 860

Arg Ile Glu Lys Glu Leu Thr Ser Ile Leu Gly Asn Gly Phe Ala Val
865                 870                 875                 880

Ile Tyr Leu Ala Ser Gln Met Leu Val Asn Arg Ser Asn Glu Arg Gly
                885                 890                 895

Tyr Leu Val Gly Ser Arg Gly Ser Val Gly Ser Ser Phe Val Ala Thr
            900                 905                 910

Met Ile Gly Ile Thr Glu Val Asn Pro Met Pro Pro His Tyr Val Cys
        915                 920                 925

Pro Ser Cys Gln His Ser Glu Phe Ile Thr Asp Gly Ser Val Gly Ser
    930                 935                 940

Gly Tyr Asp Leu Pro Asn Lys Pro Cys Pro Lys Cys Gly Thr Pro Tyr
945                 950                 955                 960

Gln Lys Asp Gly Gln Asp Ile Pro Phe Glu Thr Phe Leu Gly Phe Asp
                965                 970                 975

Gly Asp Lys Val Pro Asp Ile Asp Leu Asn Phe Ser Gly Asp Asp Gln
            980                 985                 990

Pro Ser Ala His Leu Asp Val Arg Asp Ile Phe Gly Asp Glu Tyr Ala
        995                1000                1005

Phe Arg Ala Gly Thr Val Gly Thr Val Ala Glu Lys Thr Ala Tyr Gly
   1010                1015                1020

Phe Val Lys Gly Tyr Glu Arg Asp Tyr Gly Lys Phe Tyr Arg Asp Ala
1025                1030                1035                1040
```

```
Glu Val Asp Arg Leu Ala Ala Gly Ala Ala Gly Val Lys Arg Thr Thr
                1045                1050                1055

Gly Gln His Pro Gly Gly Ile Val Val Ile Pro Asn Tyr Met Asp Val
            1060                1065                1070

Tyr Asp Phe Thr Pro Val Gln Tyr Pro Ala Asp Asp Val Thr Ala Ser
        1075                1080                1085

Trp Gln Thr Thr His Phe Asn Phe His Asp Ile Asp Glu Asn Val Leu
    1090                1095                1100

Lys Leu Asp Ile Leu Gly His Asp Asp Pro Thr Met Ile Arg Lys Leu
1105                1110                1115                1120

Gln Asp Leu Ser Gly Ile Asp Pro Ile Thr Ile Pro Ala Asp Asp Pro
                1125                1130                1135

Gly Val Met Ala Leu Phe Ser Gly Thr Glu Val Leu Gly Val Thr Pro
            1140                1145                1150

Glu Gln Ile Gly Thr Pro Thr Gly Met Leu Gly Ile Pro Glu Phe Gly
        1155                1160                1165

Thr Asn Phe Val Arg Gly Met Val Asn Glu Thr His Pro Thr Thr Phe
    1170                1175                1180

Ala Glu Leu Leu Gln Leu Ser Gly Leu Ser His Gly Thr Asp Val Trp
1185                1190                1195                1200

Leu Gly Asn Ala Gln Asp Leu Ile Lys Glu Gly Ile Ala Thr Leu Lys
                1205                1210                1215

Thr Val Ile Gly Cys Arg Asp Asp Ile Met Val Tyr Leu Met His Ala
            1220                1225                1230

Gly Leu Glu Pro Lys Met Ala Phe Thr Ile Met Glu Arg Val Arg Lys
        1235                1240                1245

Gly Leu Trp Leu Lys Ile Ser Glu Glu Glu Arg Asn Gly Tyr Ile Asp
    1250                1255                1260

Ala Met Arg Glu Asn Asn Val Pro Asp Trp Tyr Ile Glu Ser Cys Gly
1265                1270                1275                1280

Lys Ile Lys Tyr Met Phe Pro Lys Ala His Ala Ala Ala Tyr Val Leu
                1285                1290                1295

Met Ala Leu Arg Val Ala Tyr Phe Lys Val His His Pro Ile Met Tyr
            1300                1305                1310

Tyr Cys Ala Tyr Phe Ser Ile Arg Ala Lys Ala Phe Glu Leu Lys Thr
        1315                1320                1325

Met Ser Gly Gly Leu Asp Ala Val Lys Ala Arg Met Glu Asp Ile Thr
    1330                1335                1340

Ile Lys Arg Lys Asn Asn Glu Ala Thr Asn Val Glu Asn Asp Leu Phe
1345                1350                1355                1360

Thr Thr Leu Glu Ile Val Asn Glu Met Leu Glu Arg Gly Phe Lys Phe
                1365                1370                1375
```

```
Gly Lys Leu Asp Leu Tyr Lys Ser Asp Ala Ile Glu Phe Gln Ile Lys
          1380                1385                1390

Gly Asp Thr Leu Ile Pro Pro Phe Ile Ala Leu Glu Gly Leu Gly Glu
       1395                1400                1405

Asn Val Ala Lys Gln Ile Val Lys Ala Arg Gln Glu Gly Glu Phe Leu
   1410                1415                1420

Ser Lys Met Glu Leu Arg Lys Arg Gly Gly Ala Ser Ser Thr Leu Val
1425                1430                1435                1440

Glu Lys Met Asp Glu Met Gly Ile Leu Gly Asn Met Pro Glu Asp Asn
               1445                1450                1455

Gln Leu Ser Leu Phe Asp Asp Phe Phe
          1460                1465
```

The present invention also relates to the *dnaE* gene of *Streptococcus pyogenes* encoding the α-small subunit. The partial nucleotide sequence of the *dnaE* gene corresponds to SEQ. ID. No. 19 as follows:

```
atgtttgctc aacttgatac taaaactgta tactcattta tggatagttt aattgactta 60
aatcattatt ttgaacgagc aaagcaattt ggttaccaca ccataggaat catggataag 120
gataatcttt atggtgctta ccattttatt aaaggttgtc aaaaaaatgg actgcagcca 180
gttttaggtt tggaaataga gattctctat caagagcggc aggtgctcct taacttaatc 240
gcccagaata cacaaggcta tcatcagctt ttaaaaattt ccacggcaaa aatgtctggc 300
aagcttcata tggattactt ctgccaacat ttggaaggga tagcggttat tattcctagt 360
aagggttgga gcgatacatt agtggtccct tttgactact atatgggtgt tgatcagtat 420
actgatttat ctcatatgga ttctaagagg cagcttatac ccctaaggac agttcgttat 480
tttgcgcaag atgatatgga aaccctgcac atgttgcatg ccattcgaga taacctcagt 540
ctggcagaga cccctgtggt agaaagtgat caagagttag cagattgtca acaactaacc 600
gccttctatc aaacacactg ccctcaagct ctacagaatt tagaagactt agtgtcagga 660
atctattatg atttcgatac aaatttaaaa ttgcctcatt ttaatagaga taagtctgcc 720
aagcaagaat tgcaagactt gactgaggct ggtttgaagg aaaaaggatt gtggaaagag 780
ccttatcaat cgcgcttact acatgaattg gtcattattt ctgacatggg ctttgatgat 840
tatttttga ttgtgtggga tttacttcgc tttggacgca gtaaaggcta ttatatggga 900
atgggacgtg gctcggcggc aggtagtcta gtggcttatg ctctgaacat tacagggatt 960
gatccagttc aacatgattt gctatttgag cgcttttaa acaaagaacg ttatagcatg 1020
cctgatattg atatcgatct tccagatatt taccgttcag aatttctacg gtatgtccga 1080
aatcgttatg gtagcgacca ttcggcgcaa attgtgacct tttcaacctt tggccaggct 1140
attcgtgatg ttttcaaacg gttcggggtt ccagaatacg aactgactaa tctcactaaa 1200
aaaattggtt ttaaagatag cttggctact gtctatgaaa agtcaatctc ttttaggcag 1260
gttattaata gtagaactga atttcaaaag gcttttgcca ttgccaagcg tatcgaagga 1320
aatccaagac aaacgtccat tcacgcagct ggtattgtga tgagtgatga tgccttgacc 1380
aatcatattc ctctaaaatc gggcgatgac atgatgatca cccagtatga tgctcatgcg 1440
gtcgaagcta atggcctgtt aaaaatggat tttttggggt taagaaattt gacctttgtt 1500
caaaaaatgc aagagaaggt tgctaaagac tacgggtgtc agattgatat tacagccatt 1560
gatttagaag acccgcaaac gttggcactt tttgctaaag gggataccaa gggaattttc 1620
caatttgaac aaaatggtgc tattaatctt ttaaaacgga ttaagccaca acgttttgaa 1680
gaaattgttg ccactaccag tctaaataga ccaggggcaa gtgactatac cactaatttc 1740
attaaacgaa gagaaggaca agaaaaaatt gatttgattg atcctgtgat tgctcccatt 1800
ttagagccaa cttacggtat tatgctttat caagaacaag ttatgcagat tgcacaggtt 1860
tatgctggtt ttacgttagg caaggccgac ttgttaaggc gtgccatgtc taaaaaaaat 1920
ctacaagaaa tgcaaaaaat ggaagaagac tttattgctt ctgctaagca cctagggaga 1980
gctgaagaaa cagctagagg acttttaaa cggatggaaa aatttgcagg ttatggtttt 2040
aaccgcagcc atgcctttgc ctattcagct ttagcttttc aattggctta tttcaaagcc 2100
cattacccgg ctgtttttta cgatatcatg atgaattatt ctagcagtga ctatatcaca 2160
gatgctctag aatcagattt tcaagtagcg caagttacca ttaatagtat tccttacact 2220
gataaaattg aagctagcaa gatttacatg gggctgaaaa atattaaggg gttgccaagg 2280
```

```
gattttgctt attggattat cgagcaaaga ccatttaata gcgtagagga ttttctcact 2340
agaactccag aaaaatatca aaaaaaggtt ttccttgagc ctctgataaa aataggtctg 2400
tttgattgct ttgagcctaa ccgtaaaaaa attctggaca atttggatgg tttactggta 2460
tttgttaatg agcttggttc tctttttttca gattcttcct ttagttgggt agatacgaaa 2520
gattactcag taactgaaaa atattctttg gaacaggaga tcgttggagt tggcatgagc 2580
aagcatcctt taattgatat tgctgagaaa agtacccaaa cttttactcc tatttcacag 2640
ttagtcaaag aaagcgaagc agtcgtactg attcaaatag atagcattag gatcattaga 2700
accaaaacaa gtgggcagca aatggctttt ttaagtgtga atgacactaa gaaaaagctc 2760
gatgtcacac tttttccaca agagtatgcc atttataaag accaattaaa agaaggagaa 2820
ttctattact taaaaggtag aataaaagaa agagaccatc gactgcagat ggtgtgtcag 2880
caagtgcaaa tggctattag tcaaaaatat tggttattag ttgaaaacca tcagtttgat 2940
tcccaaattt ctgagatttt aggtgccttt ccaggaacga ctccagttgt tattcactat 3000
caaaaaaata aggaaacaat tgcattaact aagattcagg ttcatgtaac agagaattta 3060
aaggaaaaac ttcgtccttt tgttctgaaa acggtttttc ga                    3102
```

The encoded α-small subunit has an amino acid sequence corresponding to SEQ. ID. No. 20 as follows:

```
Met Phe Ala Gln Leu Asp Thr Lys Thr Val Tyr Ser Phe Met Asp Ser
 1               5                  10                  15

Leu Ile Asp Leu Asn His Tyr Phe Glu Arg Ala Lys Gln Phe Gly Tyr
            20                  25                  30

His Thr Ile Gly Ile Met Asp Lys Asp Asn Leu Tyr Gly Ala Tyr His
        35                  40                  45

Phe Ile Lys Gly Cys Gln Lys Asn Gly Leu Gln Pro Val Leu Gly Leu
    50                  55                  60

Glu Ile Glu Ile Leu Tyr Gln Glu Arg Gln Val Leu Leu Asn Leu Ile
65                  70                  75                  80

Ala Gln Asn Thr Gln Gly Tyr His Gln Leu Leu Lys Ile Ser Thr Ala
                85                  90                  95

Lys Met Ser Gly Lys Leu His Met Asp Tyr Phe Cys Gln His Leu Glu
            100                 105                 110

Gly Ile Ala Val Ile Ile Pro Ser Lys Gly Trp Ser Asp Thr Leu Val
        115                 120                 125

Val Pro Phe Asp Tyr Tyr Met Gly Val Asp Gln Tyr Thr Asp Leu Ser
    130                 135                 140

His Met Asp Ser Lys Arg Gln Leu Ile Pro Leu Arg Thr Val Arg Tyr
145                 150                 155                 160

Phe Ala Gln Asp Asp Met Glu Thr Leu His Met Leu His Ala Ile Arg
                165                 170                 175

Asp Asn Leu Ser Leu Ala Glu Thr Pro Val Val Glu Ser Asp Gln Glu
            180                 185                 190

Leu Ala Asp Cys Gln Gln Leu Thr Ala Phe Tyr Gln Thr His Cys Pro
        195                 200                 205

Gln Ala Leu Gln Asn L u Glu Asp Leu Val Ser Gly Ile Tyr Tyr Asp
    210                 215                 220
```

```
Phe Asp Thr Asn Leu Lys Leu Pro His Phe Asn Arg Asp Lys Ser Ala
225                 230                 235                 240

Lys Gln Glu Leu Gln Asp Leu Thr Glu Ala Gly Leu Lys Glu Lys Gly
                245                 250                 255

Leu Trp Lys Glu Pro Tyr Gln Ser Arg Leu Leu His Glu Leu Val Ile
            260                 265                 270

Ile Ser Asp Met Gly Phe Asp Asp Tyr Phe Leu Ile Val Trp Asp Leu
        275                 280                 285

Leu Arg Phe Gly Arg Ser Lys Gly Tyr Tyr Met Gly Met Gly Arg Gly
    290                 295                 300

Ser Ala Ala Gly Ser Leu Val Ala Tyr Ala Leu Asn Ile Thr Gly Ile
305                 310                 315                 320

Asp Pro Val Gln His Asp Leu Leu Phe Glu Arg Phe Leu Asn Lys Glu
                325                 330                 335

Arg Tyr Ser Met Pro Asp Ile Asp Ile Asp Leu Pro Asp Ile Tyr Arg
            340                 345                 350

Ser Glu Phe Leu Arg Tyr Val Arg Asn Arg Tyr Gly Ser Asp His Ser
        355                 360                 365

Ala Gln Ile Val Thr Phe Ser Thr Phe Gly Pro Lys Gln Ala Ile Arg
    370                 375                 380

Asp Val Phe Lys Arg Phe Gly Val Pro Glu Tyr Glu Leu Thr Asn Leu
385                 390                 395                 400

Thr Lys Lys Ile Gly Phe Lys Asp Ser Leu Ala Thr Val Tyr Glu Lys
                405                 410                 415

Ser Ile Ser Phe Arg Gln Val Ile Asn Ser Arg Thr Glu Phe Gln Lys
            420                 425                 430

Ala Phe Ala Ile Ala Lys Arg Ile Glu Gly Asn Pro Arg Gln Thr Ser
        435                 440                 445

Ile His Ala Ala Gly Ile Val Met Ser Asp Asp Ala Leu Thr Asn His
    450                 455                 460

Ile Pro Leu Lys Ser Gly Asp Asp Met Met Ile Thr Gln Tyr Asp Ala
465                 470                 475                 480

His Ala Val Glu Ala Asn Gly Leu Leu Lys Met Asp Phe Leu Gly Leu
                485                 490                 495

Arg Asn Leu Thr Phe Val Gln Lys Met Gln Glu Lys Val Ala Lys Asp
            500                 505                 510

Tyr Gly Cys Gln Ile Asp Ile Thr Ala Ile Asp Leu Glu Asp Pro Gln
        515                 520                 525

Thr Leu Ala Leu Phe Ala Lys Gly Asp Thr Lys Gly Ile Phe Gln Phe
    530                 535                 540

Glu Gln Asn Gly Ala Ile Asn Leu Leu Lys Arg Ile Lys Pro Gln Arg
545                 550                 555                 560
```

Phe Glu Glu Ile Val Ala Thr Thr Ser Leu Asn Arg Pro Gly Ala Ser
565 570 575

Asp Tyr Thr Thr Asn Phe Ile Lys Arg Arg Glu Gly Gln Glu Lys Ile
580 585 590

Asp Leu Ile Asp Pro Val Ile Ala Pro Ile Leu Glu Pro Thr Tyr Gly
595 600 605

Ile Met Leu Tyr Gln Glu Gln Val Met Gln Ile Ala Gln Val Tyr Ala
610 615 620

Gly Phe Thr Leu Gly Lys Ala Asp Leu Leu Arg Arg Ala Met Ser Lys
625 630 635 640

Lys Asn Leu Gln Glu Met Gln Lys Met Glu Glu Asp Phe Ile Ala Ser
645 650 655

Ala Lys His Leu Gly Arg Ala Glu Glu Thr Ala Arg Gly Leu Phe Lys
660 665 670

Arg Met Glu Lys Phe Ala Gly Tyr Gly Phe Asn Arg Ser His Ala Phe
675 680 685

Ala Tyr Ser Ala Leu Ala Phe Gln Leu Ala Tyr Phe Lys Ala His Tyr
690 695 700

Pro Ala Val Phe Tyr Asp Ile Met Met Asn Tyr Ser Ser Ser Asp Tyr
705 710 715 720

Ile Thr Asp Ala Leu Glu Ser Asp Phe Gln Val Ala Gln Val Thr Ile
725 730 735

Asn Ser Ile Pro Tyr Thr Asp Lys Ile Glu Ala Ser Lys Ile Tyr Met
740 745 750

Gly Leu Lys Asn Ile Lys Gly Leu Pro Arg Asp Phe Ala Tyr Trp Ile
755 760 765

Ile Glu Gln Arg Pro Phe Asn Ser Val Glu Asp Phe Leu Thr Arg Thr
770 775 780

Pro Glu Lys Tyr Gln Lys Lys Val Phe Leu Glu Pro Leu Ile Lys Ile
785 790 795 800

Gly Leu Phe Asp Cys Phe Glu Pro Asn Arg Lys Lys Ile Leu Asp Asn
805 810 815

Leu Asp Gly Leu Leu Val Phe Val Asn Glu Leu Gly Ser Leu Phe Ser
820 825 830

Asp Ser Ser Phe Ser Trp Val Asp Thr Lys Asp Tyr Ser Val Thr Glu
835 840 845

Lys Tyr Ser Leu Glu Gln Glu Ile Val Gly Val Gly Met Ser Lys His
850 855 860

Pro Leu Ile Asp Ile Ala Glu Lys Ser Thr Gln Thr Phe Thr Pro Ile
865 870 875 880

Ser Gln Leu Val Lys Glu Ser Glu Ala Val Val Leu Ile Gln Ile Asp
885 890 895

```
Ser Ile Arg Ile Ile Arg Thr Lys Thr Ser Gly Gln Gln Met Ala Phe
            900                 905                 910

Leu Ser Val Asn Asp Thr Lys Lys Lys Leu Asp Val Thr Leu Phe Pro
        915                 920                 925

Gln Glu Tyr Ala Ile Tyr Lys Asp Gln Leu Lys Glu Gly Glu Phe Tyr
    930                 935                 940

Tyr Leu Lys Gly Arg Ile Lys Glu Arg Asp His Arg Leu Gln Met Val
945                 950                 955                 960

Cys Gln Gln Val Gln Met Ala Ile Ser Gln Lys Tyr Trp Leu Leu Val
                965                 970                 975

Glu Asn His Gln Phe Asp Ser Gln Ile Ser Glu Ile Leu Gly Ala Phe
            980                 985                 990

Pro Gly Thr Thr Pro Val Val Ile His Tyr Gln Lys Asn Lys Glu Thr
        995                1000                1005

Ile Ala Leu Thr Lys Ile Gln Val Thr Glu Asn Leu Lys Glu Lys Leu
   1010                1015                1020

Arg Pro Phe Val Leu Lys Thr Val Phe Arg
1025            1030
```

The present invention also relates to the *holA* gene of *Streptococcus pyogenes* encoding the δ subunit. The *holA* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 21 as follows:

```
atgattgcga tagaaaagat tgaaaaactg agtaaagaaa atttgggtct tataaccctt 60
gtcacaggag atgacattgg tcagtatagc cagttgaaat cccgcttaat ggagcagatt 120
gcttttgata aggatgattt ggcctattct tactttgata tgtctgaggc cgcttatcag 180
gatgcagaaa tggatctagt gagcctaccc ttctttgctg agcagaaggt ggttattttt 240
gaccatttgt tagatatcac gaccaataaa aaaagtttct taaaagaaaa agacctaaag 300
gcctttgaag cctatttaga aaatccctta gagactactc gactaattat ctttgctcca 360
ggtaaattgg atagtaagag acggcttgtt aagcttttga aacgtgatgc ccttgtttta 420
gaagccaacc ctctgaaaga agcagagcta agaacttatt ttcaaaaata cagtcatcaa 480
ctgggtttag gtttcgagag tggtgccttt gaccaattac ttttgaaatc aaacgatgat 540
tttagtcaaa tcatgaaaaa catggccttt ttaaaagcct ataaaaaaac gggaaatatt 600
agcctaactg atattgagca agccattcct aaaagtttac aagataatat tttcgatctg 660
actagacttg tcctaggagg taaaattgat gcggctagag atttgattca tgatttacgg 720
ttatctggag aagatgacat taaattaatc gctatcatgc taggccaatt tcgcttattt 780
ttgcagctga ctattcttgc tagagatgta aaaaacgagc aacaactagt gattagttta 840
tcagatattc ttgggcggcg ggttaatcct taccaggtca agtatgcgtt aaaggattct 900
aggaccttat ctcttgcctt tctaacagga gcggtgaaaa ccttgattga gacagattac 960
cagataaaaa caggacttta tgagaagagt tatctagttg atattgctct cttaaaaatc 1020
atgactcact ctcaaaaa                                              1038
```

The encoded δ subunit has an amino acid sequence corresponding to SEQ. ID. No. 22 as follows:

```
Met Ile Ala Ile Glu Lys Ile Glu Lys Leu Ser Lys Glu Asn Leu Gly
 1               5                  10                  15
```

Leu Ile Thr Leu Val Thr Gly Asp Asp Ile Gly Gln Tyr Ser Gln Leu
20 25 30

Lys Ser Arg Leu Met Glu Gln Ile Ala Phe Asp Lys Asp Asp Leu Ala
35 40 45

Tyr Ser Tyr Phe Asp Met Ser Glu Ala Ala Tyr Gln Asp Ala Glu Met
50 55 60

Asp Leu Val Ser Leu Pro Phe Phe Ala Glu Gln Lys Val Val Ile Phe
65 70 75 80

Asp His Leu Leu Asp Ile Thr Thr Asn Lys Lys Ser Phe Leu Lys Glu
85 90 95

Lys Asp Leu Lys Ala Phe Glu Ala Tyr Leu Glu Asn Pro Leu Glu Thr
100 105 110

Thr Arg Leu Ile Ile Phe Ala Pro Gly Lys Leu Asp Ser Lys Arg Arg
115 120 125

Leu Val Lys Leu Leu Lys Arg Asp Ala Leu Val Leu Glu Ala Asn Pro
130 135 140

Leu Lys Glu Ala Glu Leu Arg Thr Tyr Phe Gln Lys Tyr Ser His Gln
145 150 155 160

Leu Gly Leu Gly Phe Glu Ser Gly Ala Phe Asp Gln Leu Leu Leu Lys
165 170 175

Ser Asn Asp Asp Phe Ser Gln Ile Met Lys Asn Met Ala Phe Leu Lys
180 185 190

Ala Tyr Lys Lys Thr Gly Asn Ile Ser Leu Thr Asp Ile Glu Gln Ala
195 200 205

Ile Pro Lys Ser Leu Gln Asp Asn Ile Phe Asp Leu Thr Arg Leu Val
210 215 220

Leu Gly Gly Lys Ile Asp Ala Ala Arg Asp Leu Ile His Asp Leu Arg
225 230 235 240

Leu Ser Gly Glu Asp Asp Ile Lys Leu Ile Ala Ile Met Leu Gly Gln
245 250 255

Phe Arg Leu Phe Leu Gln Leu Thr Ile Leu Ala Arg Asp Val Lys Asn
260 265 270

Glu Gln Gln Leu Val Ile Ser Leu Ser Asp Ile Leu Gly Arg Arg Val
275 280 285

Asn Pro Tyr Gln Val Lys Tyr Ala Leu Lys Asp Ser Arg Thr Leu Ser
290 295 300

Leu Ala Phe Leu Thr Gly Ala Val Lys Thr Leu Ile Glu Thr Asp Tyr
305 310 315 320

Gln Ile Lys Thr Gly Leu Tyr Glu Lys Ser Tyr Leu Val Asp Ile Ala
325 330 335

Leu Leu Lys Ile Met Thr His Ser Gln Lys
340 345

The present invention also relates to the *holB* gene of *Streptococcus pyogenes* encoding the δ' subunit. The *holB* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 23 as follows:

```
atggatttag cgcaaaaagc tcctaacgtt tatcaagctt ttcagacaat tttaaagaaa 60
gaccgtctga atcatgctta tctttttttcg ggtgattttg ctaatgaaga aatggctctt 120
tttttagcta aggtcatctt ttgtgaacag aaaaaggatc agacgccctg cgggcattgt 180
cgctcttgtc aattgattga acaaggagat tttgccgatg tgacggtatt ggaaccaaca 240
gggcaagtga ttaaaacgga tgtggtcaaa gaaatgatgg ctaacttttc tcagacagga 300
tatgaaaaca aacgacaagt ttttattatc aaagattgtg acaaaatgca tatcaatgcc 360
gctaatagct tgctaaaata cattgaggag cctcagggag aagcttacat atttttattg 420
accaatgatg ataacaaagt gcttccgacc attaaaagtc ggacacaggt ttttcagttt 480
cctaaaaacg aagcctatct ttaccaattg gcacaagaaa agggattatt aaaccatcag 540
gctaagctag tagccaaact tgccacaaac accagtcatc tagaacgtct gttgcaaacg 600
agcaagcttt tagaactgat aactcaagca gagcgttttg tatctatttg gctgaaagat 660
cagttgcagg catatttagc gttgaaccgt ctggtacagt tagcaactga aaaagaagaa 720
caagatttag ttttgaccct tttgaccttg ctcttggcaa gagagcgtgc gcaaacgcct 780
ttgacacaat tggaggctgt ctatcaggct aggctcatgt ggcagagcaa tgttaatttt 840
caaaacacat tagaatatat ggtgatgtca gaa                              873
```

The encoded δ' subunit has an amino acid sequence corresponding to SEQ. ID. No. 24 as follows:

```
Met Asp Leu Ala Gln Lys Ala Pro Asn Val Tyr Gln Ala Phe Gln Thr
  1               5                  10                  15

Ile Leu Lys Lys Asp Arg Leu Asn His Ala Tyr Leu Phe Ser Gly Asp
             20                  25                  30

Phe Ala Asn Glu Glu Met Ala Leu Phe Leu Ala Lys Val Ile Phe Cys
         35                  40                  45

Glu Gln Lys Lys Asp Gln Thr Pro Cys Gly His Cys Arg Ser Cys Gln
     50                  55                  60

Leu Ile Glu Gln Gly Asp Phe Ala Asp Val Thr Val Leu Glu Pro Thr
 65                  70                  75                  80

Gly Gln Val Ile Lys Thr Asp Val Val Lys Glu Met Met Ala Asn Phe
                 85                  90                  95

Ser Gln Thr Gly Tyr Glu Asn Lys Arg Gln Val Phe Ile Ile Lys Asp
            100                 105                 110

Cys Asp Lys Met His Ile Asn Ala Ala Asn Ser Leu Leu Lys Tyr Ile
        115                 120                 125

Glu Glu Pro Gln Gly Glu Ala Tyr Ile Phe Leu Leu Thr Asn Asp Asp
    130                 135                 140

Asn Lys Val Leu Pro Thr Ile Lys Ser Arg Thr Gln Val Phe Gln Phe
145                 150                 155                 160

Pro Lys Asn Glu Ala Tyr Leu Tyr Gln Leu Ala Gln Glu Lys Gly Leu
                165                 170                 175

Leu Asn His Gln Ala Lys Leu Val Ala Lys Leu Ala Thr Asn Thr Ser
            180                 185                 190
```

```
His Leu Glu Arg Leu Leu Gln Thr Ser Lys Leu Leu Glu Leu Ile Thr
        195                 200                 205

Gln Ala Glu Arg Phe Val Ser Ile Trp Leu Lys Asp Gln Leu Gln Ala
    210                 215                 220

Tyr Leu Ala Leu Asn Arg Leu Val Gln Leu Ala Thr Glu Lys Glu Glu
225                 230                 235                 240

Gln Asp Leu Val Leu Thr Leu Leu Thr Leu Leu Leu Ala Arg Glu Arg
                245                 250                 255

Ala Gln Thr Pro Leu Thr Gln Leu Glu Ala Val Tyr Gln Ala Arg Leu
            260                 265                 270

Met Trp Gln Ser Asn Val Asn Phe Gln Asn Thr Leu Glu Tyr Met Val
        275                 280                 285

Met Ser Glu
    290
```

The present invention also relates to the *dnaX* gene of *Streptococcus pyogenes* encoding the τ subunit. The *dnaX* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 25 as follows:

```
atgtatcaag ctctttatcg gaaataccgg agccaaacgt ttgacgaaat ggtgggacaa 60
tcggttattt ccacaacttt aaagcaggca gttgaatctg gcaagattag ccatgcttat 120
ctttttttcag gtcctagagg gactgggaaa acaagtgcgg caaagatttt tgcaaaggcc 180
atgaattgtc ctaaccaagt cgatggtgaa ccctgtaatc aatgcgatat ttgccgagat 240
atcacgaatg gaagcttgga agatgtgatt gaaattgatg ctgcctcgaa taatggtgtt 300
gatgaaattc gtgacattcg agacaaatca acctatgcgc caagtcgtgc gacttacaag 360
gtttatatta ttgatgaggt tcacatgtta tcaacagggg cttttaatgc gcttttgaaa 420
actttggaag aaccgacaga atgttgtctt tatcttggca acaacggaat gcataaaatt 480
ccagccacta ttttatctcg tgtgcaacgc tttgaattca aagctattaa gcaaaaagct 540
attcgagagc atttagcctg ggttttggac aaagaaggta ttgcctatga ggtggatgct 600
ttaaatctca ttgcaaggcg agcagaagga ggcatgcgtg atgctttatc tattttagat 660
caggctttga gcttgtcacc agataatcag gtcgccattg caattgccga agaaattaca 720
ggttctattt ccatacttgc tctgggtgac tatgttcgat atgtctccca agaacaggct 780
acgcaagctc tggcagcctt agagaccatt tatgatagtg ggaagagcat gagccgcttt 840
gcgacagatt tattgaccta tctgcgtgat ttattggtgg ttaaagctgg cggcgacaat 900
caacgtcagt cagctgtttt tgataccaat ttgtctctct cgatagatcg tatattccaa 960
atgataacag ttgttactag tcatctccct gaaatcaaaa agggaaccca tcctcggatt 1020
tatgccgaaa tgatgactat ccaattagct cagaaagagc agattttgtc ccaagtaaac 1080
ttgtcaggag agttaatctc agagattgaa acgctcaaaa atgagttggc acaacttaaa 1140
caacaattgt cgcagctcca atcgcgtcct gattcactgg caagatctga taaaacgaaa 1200
cctaaaacca caagctacag ggttgatcgg gttaccattt tgaaaatcat ggaagaaacg 1260
gttcgaaata gccaacaatc tcgacaatat ctagatgctc taaaaaatgc ttggaatgaa 1320
attctagata acatttctgc ccaagacaga gccttattga tgggctctga gcctgtctta 1380
gcaaatagtg agaatgcgat tttggctttc gaggctgcct ttaatgcaga acaagtcatg 1440
agccgaaata atcttaatga tatgtttggt aacattatga gtaaagctgc tggtttttct 1500
cccaatattc tggcagtacc aaggacagat tttcagcata ttcgtaagga atttgctcag 1560
caaatgaaat cgcaaaaaga cagtgttcaa gaagaacaag aagtagcgct tgatattcca 1620
gaagggtttg atttttgct cgataaaata aatactattg acgac                  1665
```

The encoded τ subunit has an amino acid sequence corresponding to SEQ. ID. No. 26 as follows:

```
Met Tyr Gln Ala Leu Tyr Arg Lys Tyr Arg Ser Gln Thr Phe Asp Glu
 1               5                  10                  15

Met Val Gly Gln Ser Val Ile Ser Thr Thr Leu Lys Gln Ala Val Glu
            20                  25                  30

Ser Gly Lys Ile Ser His Ala Tyr Leu Phe Ser Gly Pro Arg Gly Thr
        35                  40                  45

Gly Lys Thr Ser Ala Ala Lys Ile Phe Ala Lys Ala Met Asn Cys Pro
    50                  55                  60

Asn Gln Val Asp Gly Glu Pro Cys Asn Gln Cys Asp Ile Cys Arg Asp
65                  70                  75                  80

Ile Thr Asn Gly Ser Leu Glu Asp Val Ile Glu Ile Asp Ala Ala Ser
                85                  90                  95

Asn Asn Gly Val Asp Glu Ile Arg Asp Ile Arg Asp Lys Ser Thr Tyr
            100                 105                 110

Ala Pro Ser Arg Ala Thr Tyr Lys Val Tyr Ile Ile Asp Glu Val His
        115                 120                 125

Met Leu Ser Thr Gly Ala Phe Asn Ala Leu Leu Lys Thr Leu Glu Glu
    130                 135                 140

Pro Thr Glu Asn Val Phe Ile Leu Ala Thr Thr Glu Leu His Lys Ile
145                 150                 155                 160

Pro Ala Thr Ile Leu Ser Arg Val Gln Arg Phe Glu Phe Lys Ala Ile
                165                 170                 175

Lys Gln Lys Ala Ile Arg Glu His Leu Ala Trp Val Leu Asp Lys Glu
            180                 185                 190

Gly Ile Ala Tyr Glu Val Asp Ala Leu Asn Leu Ile Ala Arg Arg Ala
        195                 200                 205

Glu Gly Gly Met Arg Asp Ala Leu Ser Ile Leu Asp Gln Ala Leu Ser
    210                 215                 220

Leu Ser Pro Asp Asn Gln Val Ala Ile Ala Ile Ala Glu Glu Ile Thr
225                 230                 235                 240

Gly Ser Ile Ser Ile Leu Ala Leu Gly Asp Tyr Val Arg Tyr Val Ser
                245                 250                 255

Gln Glu Gln Ala Thr Gln Ala Leu Ala Ala Leu Glu Thr Ile Tyr Asp
            260                 265                 270

Ser Gly Lys Ser Met Ser Arg Phe Ala Thr Asp Leu Leu Thr Tyr Leu
        275                 280                 285

Arg Asp Leu Leu Val Val Lys Ala Gly Gly Asp Asn Gln Arg Gln Ser
    290                 295                 300

Ala Val Phe Asp Thr Asn Leu Ser Leu Ser Ile Asp Arg Ile Phe Gln
305                 310                 315                 320

Met Ile Thr Val Val Thr Ser His Leu Pro Glu Ile Lys Lys Gly Thr
                325                 330                 335
```

```
His Pro Arg Ile Tyr Ala Glu Met Met Thr Ile Gln Leu Ala Gln Lys
            340                 345                 350

Glu Gln Ile Leu Ser Gln Val Asn Leu Ser Gly Glu Leu Ile Ser Glu
        355                 360                 365

Ile Glu Thr Leu Lys Asn Glu Leu Ala Gln Leu Lys Gln Gln Leu Ser
    370                 375                 380

Gln Leu Gln Ser Arg Pro Asp Ser Leu Ala Arg Ser Asp Lys Thr Lys
385                 390                 395                 400

Pro Lys Thr Thr Ser Tyr Arg Val Asp Arg Val Thr Ile Leu Lys Ile
                405                 410                 415

Met Glu Glu Thr Val Arg Asn Ser Gln Gln Ser Arg Gln Tyr Leu Asp
            420                 425                 430

Ala Leu Lys Asn Ala Trp Asn Glu Ile Leu Asp Asn Ile Ser Ala Gln
        435                 440                 445

Asp Arg Ala Leu Leu Met Gly Ser Glu Pro Val Leu Ala Asn Ser Glu
    450                 455                 460

Asn Ala Ile Leu Ala Phe Glu Ala Ala Phe Asn Ala Glu Gln Val Met
465                 470                 475                 480

Ser Arg Asn Asn Leu Asn Asp Met Phe Gly Asn Ile Met Ser Lys Ala
                485                 490                 495

Ala Gly Phe Ser Pro Asn Ile Leu Ala Val Pro Arg Thr Asp Phe Gln
            500                 505                 510

His Ile Arg Lys Glu Phe Ala Gln Gln Met Lys Ser Gln Lys Asp Ser
        515                 520                 525

Val Gln Glu Glu Gln Glu Val Ala Leu Asp Ile Pro Glu Gly Phe Asp
    530                 535                 540

Phe Leu Leu Asp Lys Ile Asn Thr Ile Asp Asp
545                 550                 555
```

The present invention also relates to the *dnaN* gene of *Streptococcus pyogenes* encoding the β subunit. The *dnaN* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 27 as follows:

```
atgattcaat tttcaattaa tcgcacatta tttattcatg ctttaaatac aactaaacgt 60
gctattagca ctaaaaatgc cattcctatt ctttcatcaa taaaaattga agtcacttct 120
acaggagtaa ctttaacagg gtctaacggt caaatatcaa ttgaaaacac tattcctgta 180
agtaatgaaa atgctggttt gctaattacc tctccaggag ctattttatt agaagctagt 240
ttttttatta atattatttc aagtttgcca gatattagta taaatgttaa agaaattgaa 300
caacaccaag ttgttttaac cagtggtaaa tcagagatta ccttaaaagg aaaagatgtt 360
gaccagtatc ctcgtctaca agaagtatca acagaaaatc ctttgatttt aaaaacaaaa 420
ttattgaagt ctattattgc tgaaacagct tttgcagcca gtttacaaga aagtcgtcct 480
attttaacag gagttcatat tgtattaagt aatcataaag attttaaagc agtagcgact 540
gactctcatc gtatgagcca acgtttaatc actttggaca atacttcagc agatttgatg 600
gtagttcttc caagtaaatc tttgagagaa ttttcagcag tatttacaga tgatattgag 660
accgttgagg tatttttctc accaagccaa atcttgttca gaagtgaaca catttctttt 720
```

```
tatacacgcc tcttagaagg aaattatccc gatacagacc gtttattaat gacagaattt 780
gagacggagg ttgttttcaa tacccaatcc cttcgccacg ctatggaacg tgccttcttg 840
atttctaatg ctactcaaaa tggtactgtt aagcttgaga ttactcaaaa tcatatttca 900
gctcatgtta actcacctga ggttggtaag gtaaacgagg atttagatat tgttagtcag 960
tctggtagtg atttaactat cagcttcaat ccaacttacc ttattgagtc tttaaaagct 1020
attaaaagtg aaacagtaaa aattcatttc ttatcaccag ttcgaccatt caccctaaca 1080
ccaggcgatg aggaagaaag ttttatccaa ttaattacac cagtacgaac aaac       1134
```

The encoded β subunit has an amino acid sequence corresponding to SEQ. ID. No. 28 as follows:

```
Met Ile Gln Phe Ser Ile Asn Arg Thr Leu Phe Ile His Ala Leu Asn
 1               5                  10                  15

Thr Thr Lys Arg Ala Ile Ser Thr Lys Asn Ala Ile Pro Ile Leu Ser
            20                  25                  30

Ser Ile Lys Ile Glu Val Thr Ser Thr Gly Val Thr Leu Thr Gly Ser
        35                  40                  45

Asn Gly Gln Ile Ser Ile Glu Asn Thr Ile Pro Val Ser Asn Glu Asn
    50                  55                  60

Ala Gly Leu Leu Ile Thr Ser Pro Gly Ala Ile Leu Leu Glu Ala Ser
65                  70                  75                  80

Phe Phe Ile Asn Ile Ile Ser Ser Leu Pro Asp Ile Ser Ile Asn Val
                85                  90                  95

Lys Glu Ile Glu Gln His Gln Val Val Leu Thr Ser Gly Lys Ser Glu
            100                 105                 110

Ile Thr Leu Lys Gly Lys Asp Val Asp Gln Tyr Pro Arg Leu Gln Glu
        115                 120                 125

Val Ser Thr Glu Asn Pro Leu Ile Leu Lys Thr Lys Leu Leu Lys Ser
    130                 135                 140

Ile Ile Ala Glu Thr Ala Phe Ala Ala Ser Leu Gln Glu Ser Arg Pro
145                 150                 155                 160

Ile Leu Thr Gly Val His Ile Val Leu Ser Asn His Lys Asp Phe Lys
                165                 170                 175

Ala Val Ala Thr Asp Ser His Arg Met Ser Gln Arg Leu Ile Thr Leu
            180                 185                 190

Asp Asn Thr Ser Ala Asp Leu Met Val Val Leu Pro Ser Lys Ser Leu
        195                 200                 205

Arg Glu Phe Ser Ala Val Phe Thr Asp Asp Ile Glu Thr Val Glu Val
    210                 215                 220

Phe Phe Ser Pro Ser Gln Ile Leu Phe Arg Ser Glu His Ile Ser Phe
225                 230                 235                 240

Tyr Thr Arg Leu Leu Glu Gly Asn Tyr Pro Asp Thr Asp Arg Leu Leu
                245                 250                 255

Met Thr Glu Phe Glu Thr Glu Val Val Phe Asn Thr Gln Ser Leu Arg
            260                 265                 270
```

```
His Ala Met Glu Arg Ala Phe Leu Ile Ser Asn Ala Thr Gln Asn Gly
        275                 280                 285

Thr Val Lys Leu Glu Ile Thr Gln Asn His Ile Ser Ala His Val Asn
    290                 295                 300

Ser Pro Glu Val Gly Lys Val Asn Glu Asp Leu Asp Ile Val Ser Gln
305                 310                 315                 320

Ser Gly Ser Asp Leu Thr Ile Ser Phe Asn Pro Thr Tyr Leu Ile Glu
                325                 330                 335

Ser Leu Lys Ala Ile Lys Ser Glu Thr Val Lys Ile His Phe Leu Ser
            340                 345                 350

Pro Val Arg Pro Phe Thr Leu Thr Pro Gly Asp Glu Glu Glu Ser Phe
        355                 360                 365

Ile Gln Leu Ile Thr Pro Val Arg Thr Asn
    370                 375
```

The present invention also relates to the *ssb* gene of *Streptococcus pyogenes* encoding the single strand-binding protein (SSB). The *ssb* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 29 as follows:

```
atgattaata atgtagtact agttggtcgc atgaccaagg atgcagaact tcgttacaca 60
ccaagtcaag tagctgtggc taccttcaca cttgctgtta accgtacctt taaaagccaa 120
aatggtgaac gcgaggcaga tttcattaac tgtgtgatct ggcgtcaacc ggctgaaaat 180
ttagcgaact gggctaaaaa aggtgctttg atcggagtta cgggtcgtat tcatacacgt 240
aactacgaaa accaacaagg acaacgtgtc tatgtaacag aagttgttgc agataatttc 300
caaatgttgg aaagtcgtgc tacacgtgaa ggtggctcaa ctggctcatt taatggtggt 360
tttaacaata acacttcatc atcaaacagt tactcagcgc ctgcacaaca aacgcctaac 420
tttggaagag atgatagccc atttgggaac tcaaacccga tggatatctc agatgacgat 480
cttccattct ag                                                    492
```

The encoded SSB protein has an amino acid sequence corresponding to SEQ. ID. No. 30 as follows:

```
Met Ile Asn Asn Val Val Leu Val Gly Arg Met Thr Lys Asp Ala Glu
  1               5                  10                  15

Leu Arg Tyr Thr Pro Ser Gln Val Ala Val Ala Thr Phe Thr Leu Ala
            20                  25                  30

Val Asn Arg Thr Phe Lys Ser Gln Asn Gly Glu Arg Glu Ala Asp Phe
        35                  40                  45

Ile Asn Cys Val Ile Trp Arg Gln Pro Ala Glu Asn Leu Ala Asn Trp
    50                  55                  60

Ala Lys Lys Gly Ala Leu Ile Gly Val Thr Gly Arg Ile Gln Thr Arg
65                  70                  75                  80

Asn Tyr Glu Asn Gln Gln Gly Gln Arg Val Tyr Val Thr Glu Val Val
                85                  90                  95
```

```
Ala Asp Asn Phe Gln Met Leu Glu Ser Arg Ala Thr Arg Glu Gly Gly
            100                 105                 110

Ser Thr Gly Ser Phe Asn Gly Gly Phe Asn Asn Asn Thr Ser Ser Ser
        115                 120                 125

Asn Ser Tyr Ser Ala Pro Ala Gln Gln Thr Pro Asn Phe Gly Arg Asp
    130                 135                 140

Asp Ser Pro Phe Gly Asn Ser Asn Pro Met Asp Ile Ser Asp Asp Asp
145                 150                 155                 160

Leu Pro Phe
```

The present invention also relates to the *dnaG* gene of *Streptococcus pyogenes* encoding the primase. The *dnaG* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 31 as follows:

```
atgggatttt tatggggagg tgacgatttg gcaattgaca aagaaatgat ttcccaagta 60
aaaaatagcg ttaatattgt cgatgtcatt ggagaagtgg tcaaactttc ccgatcaggg 120
cggcattacc tcgggctttg cccatttcat aaggaaaaga cacctctttt taatgttgtt 180
gaagacagac aatttttttca ctgctttggc tgtggaaaat caggggatgt ttttaaattt 240
attgaggaat accgccaagt ccccttctta gaaagtgttc agattattgc cgataagact 300
ggtatgtcgc ttaatatacc gccaagtcag gcagtacttg ctagccaaca caagcaccct 360
aatcacgctt tgatgacact tcatgaggat gctgctaaat tttaccatgc agttttgatg 420
accactacca ttggtcaaga agctaggaag tacctttacc agagaggctt ggatgaccaa 480
ttaattgagc atttcaatat tggtttagcc ccagatgagt cagattatct ttatcaagct 540
ctttctaaaa aatacgagga aggtcaattg gttgcttcag gattgtttca cttgtccgat 600
caatccaata ccatttacga cgcctttcga aatcgtatca tgtttccctt atcagatgac 660
cgagggcata ttattgcctt ttcaggacgt atctggacgg cagctgatat ggaaaagaga 720
caggcaaagt ataaaaattc aagaggaaca gttcttttta acaaatctta tgaattgtat 780
catctggaca aggcaaggcc tgttattgcc aaaacccatg aagtgtttct aatggaaggg 840
tttatggacg tgattgccgc ttaccgttcc ggctatgaaa atgctgttgc ttcaatgggg 900
acggcattga ctcaagaaca tgtcaatcac cttaagcaag tcactaaaaa agttgttttg 960
atttatgatg gtgacgatgc tggacaacat gctattgcaa aatcactaga attgcttaaa 1020
gattttgttg tcgaaattgt cagaatcccc aataaaatgg atcctgacga atttgtacaa 1080
cggcattccc cagaagcatt tgcagatttg cttaagcagt cacggatcag tagtgttgaa 1140
ttttttattg attacctaaa acctactaat gtagacaatt tgcaatcaca aattgtttat 1200
gtggagaaaa tggcaccatt gattgctcaa tcaccatcca tcacagctca acattcgtat 1260
attaacaaga ttgctgattt gttgccaaac tttgactatt ttcaagtaga acaatcagta 1320
aatgcattaa ggattcaaga taggcaaaaa catcaaggtc aaatagctca agccgtcagc 1380
aatcttgtga ccttaccaat gccaaaaagt ttgacagcta ttgctaagac agaaagtcat 1440
ctcatgcatc ggctcttaca tcatgactat ttattaaatg aatttcgaca tcgtgatgat 1500
ttttattttg atacctctac cttagaatta ctttatcaac ggctgaagca acaaggacac 1560
attacatctt atgatttgtc agagatgtca gaggaagtta accgtgctta ttacaatgtt 1620
ttagaagaaa accttcccaa agaagtagct cttggtgaga ttgatgatat tttatccaaa 1680
cgtgccaaac ttttagcaga gcgcgatctt cacaaacaag ggaaaaaagt tagagaatct 1740
agtaacaaag gcgatcatca agcggctcta gaagtactag aacattttat tgcgcagaaa 1800
cgaaaaatgg aatag                                                  1815
```

The encoded primase has an amino acid sequence corresponding to SEQ. ID. No. 32 as follows:

```
Met Gly Phe Leu Trp Gly Gly Asp Asp Leu Ala Ile Asp Lys Glu Met
 1               5                  10                  15

Ile Ser Gln Val Lys Asn Ser Val Asn Ile Val Asp Val Ile Gly Glu
            20                  25                  30

Val Val Lys Leu Ser Arg Ser Gly Arg His Tyr Leu Gly Leu Cys Pro
        35                  40                  45

Phe His Lys Glu Lys Thr Pro Ser Phe Asn Val Val Glu Asp Arg Gln
    50                  55                  60

Phe Phe His Cys Phe Gly Cys Gly Lys Ser Gly Asp Val Phe Lys Phe
65                  70                  75                  80

Ile Glu Glu Tyr Arg Gln Val Pro Phe Leu Glu Ser Val Gln Ile Ile
                85                  90                  95

Ala Asp Lys Thr Gly Met Ser Leu Asn Ile Pro Pro Ser Gln Ala Val
            100                 105                 110

Leu Ala Ser Gln His Lys His Pro Asn His Ala Leu Met Thr Leu His
        115                 120                 125

Glu Asp Ala Ala Lys Phe Tyr His Ala Val Leu Met Thr Thr Thr Ile
    130                 135                 140

Gly Gln Glu Ala Arg Lys Tyr Leu Tyr Gln Arg Gly Leu Asp Asp Gln
145                 150                 155                 160

Leu Ile Glu His Phe Asn Ile Gly Leu Ala Pro Asp Glu Ser Asp Tyr
                165                 170                 175

Leu Tyr Gln Ala Leu Ser Lys Lys Tyr Glu Glu Gly Gln Leu Val Ala
            180                 185                 190

Ser Gly Leu Phe His Leu Ser Asp Gln Ser Asn Thr Ile Tyr Asp Ala
        195                 200                 205

Phe Arg Asn Arg Ile Met Phe Pro Leu Ser Asp Asp Arg Gly His Ile
    210                 215                 220

Ile Ala Phe Ser Gly Arg Ile Trp Thr Ala Ala Asp Met Glu Lys Arg
225                 230                 235                 240

Gln Ala Lys Tyr Lys Asn Ser Arg Gly Thr Val Leu Phe Asn Lys Ser
                245                 250                 255

Tyr Glu Leu Tyr His Leu Asp Lys Ala Arg Pro Val Ile Ala Lys Thr
            260                 265                 270

His Glu Val Phe Leu Met Glu Gly Phe Met Asp Val Ile Ala Ala Tyr
        275                 280                 285

Arg Ser Gly Tyr Glu Asn Ala Val Ala Ser Met Gly Thr Ala Leu Thr
    290                 295                 300

Gln Glu His Val Asn His Leu Lys Gln Val Thr Lys Lys Val Val Leu
305                 310                 315                 320

Ile Tyr Asp Gly Asp Asp Ala Gly Gln His Ala Ile Ala Lys Ser Leu
                325                 330                 335
```

```
Glu Leu Leu Lys Asp Phe Val Val Glu Ile Val Arg Ile Pro Asn Lys
            340                 345                 350

Met Asp Pro Asp Glu Phe Val Gln Arg His Ser Pro Glu Ala Phe Ala
        355                 360                 365

Asp Leu Leu Lys Gln Ser Arg Ile Ser Ser Val Glu Phe Phe Ile Asp
    370                 375                 380

Tyr Leu Lys Pro Thr Asn Val Asp Asn Leu Gln Ser Gln Ile Val Tyr
385                 390                 395                 400

Val Glu Lys Met Ala Pro Leu Ile Ala Gln Ser Pro Ser Ile Thr Ala
                405                 410                 415

Gln His Ser Tyr Ile Asn Lys Ile Ala Asp Leu Leu Pro Asn Phe Asp
            420                 425                 430

Tyr Phe Gln Val Glu Gln Ser Val Asn Ala Leu Arg Ile Gln Asp Arg
        435                 440                 445

Gln Lys His Gln Gly Gln Ile Ala Gln Ala Val Ser Asn Leu Val Thr
    450                 455                 460

Leu Pro Met Pro Lys Ser Leu Thr Ala Ile Ala Lys Thr Glu Ser His
465                 470                 475                 480

Leu Met His Arg Leu Leu His His Asp Tyr Leu Leu Asn Glu Phe Arg
                485                 490                 495

His Arg Asp Asp Phe Tyr Phe Asp Thr Ser Thr Leu Glu Leu Leu Tyr
            500                 505                 510

Gln Arg Leu Lys Gln Gln Gly His Ile Thr Ser Tyr Asp Leu Ser Glu
        515                 520                 525

Met Ser Glu Glu Val Asn Arg Ala Tyr Tyr Asn Val Leu Glu Glu Asn
    530                 535                 540

Leu Pro Lys Glu Val Ala Leu Gly Glu Ile Asp Asp Ile Leu Ser Lys
545                 550                 555                 560

Arg Ala Lys Leu Leu Ala Glu Arg Asp Leu His Lys Gln Gly Lys Lys
                565                 570                 575

Val Arg Glu Ser Ser Asn Lys Gly Asp His Gln Ala Ala Leu Glu Val
            580                 585                 590

Leu Glu His Phe Ile Ala Gln Lys
        595                 600
```

The present invention also relates to the *dnaB* gene of *Streptococcus pyogenes* encoding DnaB. The *dnaB* gene has a nucleotide sequence which corresponds to SEQ. ID. No. 33 as follows:

```
atgaggttgc ctgaagtagc tgaattacga gttcaacccc aagatttact agcagagcaa 60
tctgttcttg ggtcaatctt tatctcacct gataagctga ttgcagtgag agaatttatc 120
agtccagacg atttttataa gtacgctcat aaaattatct ttcgggcaat gattaccctc 180
agcgatcgta atgatgccat tgatgcaacc actataagaa caatcctaga tgatcaagat 240
```

```
gatctgcaaa gtattggtgg cttatcctat attgttgaac tagttaatag tgtcccaact 300
agtgctaatg cagaatatta tgctaaaatt gtagctgaga aagctatgtt gcgtgatatt 360
attgctaggt tgacagaatc tgtcaaccta gcttatgatg aaattttaaa accagaagag 420
gttatcgctg gagttgagag agctttaatt gaactcaatg aacatagtaa tcgtagtggg 480
tttcgcaaaa tttcagatgt gctaaaagtt aattacgagg ctttagaagc acgttctaag 540
cagacttcaa atgttacagg tttaccaact ggttttagag accttgacaa gattacaaca 600
ggtttacacc cagatcaatt agttatttta gctgctcggc cagcagtggg gaagactgcc 660
tttgttctta atattgcgca aaatgtgggg actaagcaaa aaaagactgt tgctattttt 720
tctttggaaa tgggtgctga aagtttagta gatcgtatgc ttgcagcaga aggaatggtt 780
gattcgcaca gtttaagaac agggcaactc acagatcagg attggaataa tgtaacaatt 840
gctcagggag ctttggcaga agcaccgatt tatattgacg atacgcccgg gattaaaatt 900
actgaaatcc gcgcaagatc acggaaattg tctcaagaag tggatggtgg tttaggtctc 960
attgtaattg actacttaca gttgattaca ggaactaaac ccgaaaatcg tcagcaagag 1020
gtttcagata tttcaagaca gcttaaaatc ctagctaaag aattgaaagt accagttatt 1080
gccctaagtc agctttctcg tggcgttgag caaaggcaag ataaacgacc agttttatca 1140
gatattcgtg aatcaggatc tattgagcag gatgccgata ttgtagcctt cttataccgg 1200
gacgattatt accgtaaaga atgtgatgat gctgaagaag ctgttgaaga taacacaatt 1260
gaagttatcc tcgagaaaaa tagagctggg gcgcgtggaa cagtcaaact gatgttccaa 1320
aaagaataca acaaattctc aagtatagcc cagtttgaag aaagataa              1368
```

The encoded DnaB has an amino acid sequence corresponding to SEQ. ID. No. 34 as follows:

```
Met Arg Leu Pro Glu Val Ala Glu Leu Arg Val Gln Pro Gln Asp Leu
 1               5                  10                  15

Leu Ala Glu Gln Ser Val Leu Gly Ser Ile Phe Ile Ser Pro Asp Lys
            20                  25                  30

Leu Ile Ala Val Arg Glu Phe Ile Ser Pro Asp Asp Phe Tyr Lys Tyr
        35                  40                  45

Ala His Lys Ile Ile Phe Arg Ala Met Ile Thr Leu Ser Asp Arg Asn
    50                  55                  60

Asp Ala Ile Asp Ala Thr Thr Ile Arg Thr Ile Leu Asp Asp Gln Asp
65                  70                  75                  80

Asp Leu Gln Ser Ile Gly Gly Leu Ser Tyr Ile Val Glu Leu Val Asn
                85                  90                  95

Ser Val Pro Thr Ser Ala Asn Ala Glu Tyr Tyr Ala Lys Ile Val Ala
            100                 105                 110

Glu Lys Ala Met Leu Arg Asp Ile Ile Ala Arg Leu Thr Glu Ser Val
        115                 120                 125

Asn Leu Ala Tyr Asp Glu Ile Leu Lys Pro Glu Glu Val Ile Ala Gly
    130                 135                 140

Val Glu Arg Ala Gln Gly Ala Leu Ala Glu Ala Pro Ile Tyr Ile Asp
145                 150                 155                 160

Asp Thr Pro Gly Ile Lys Ile Ala Leu Ile Glu Leu Asn Glu His Ser
                165                 170                 175

Asn Arg Ser Gly Phe Arg Lys Ile Ser Asp Val Leu Lys Val Asn Tyr
            180                 185                 190

Glu Ala Leu Glu Ala Arg Ser Lys Gln Thr Ser Asn Val Thr Gly Leu
        195                 200                 205
```

```
Pro Thr Gly Phe Arg Asp Leu Asp Lys Ile Thr Thr Gly Leu His Pro
    210                 215                 220

Asp Gln Leu Val Ile Leu Ala Ala Arg Pro Ala Val Gly Lys Thr Ala
225                 230                 235                 240

Phe Val Leu Asn Ile Ala Gln Asn Val Gly Thr Lys Gln Lys Lys Thr
                245                 250                 255

Val Ala Ile Phe Ser Leu Glu Met Gly Ala Glu Ser Leu Val Asp Arg
            260                 265                 270

Met Leu Ala Ala Glu Gly Met Val Asp Ser His Ser Leu Arg Thr Gly
        275                 280                 285

Gln Leu Thr Asp Gln Asp Trp Asn Asn Val Thr Ile Thr Glu Ile Arg
    290                 295                 300

Ala Arg Ser Arg Lys Leu Ser Gln Glu Val Asp Gly Gly Leu Gly Leu
305                 310                 315                 320

Ile Val Ile Asp Tyr Leu Gln Leu Ile Thr Gly Thr Lys Pro Glu Asn
                325                 330                 335

Arg Gln Gln Glu Val Ser Asp Ile Ser Arg Gln Leu Lys Ile Leu Ala
            340                 345                 350

Lys Glu Leu Lys Val Pro Val Ile Ala Leu Ser Gln Leu Ser Arg Gly
        355                 360                 365

Val Glu Gln Arg Gln Asp Lys Arg Pro Val Leu Ser Asp Ile Arg Glu
    370                 375                 380

Ser Gly Ser Ile Glu Gln Asp Ala Asp Ile Val Ala Phe Leu Tyr Arg
385                 390                 395                 400

Asp Asp Tyr Tyr Arg Lys Glu Cys Asp Asp Ala Glu Glu Ala Val Glu
                405                 410                 415

Asp Asn Thr Ile Glu Val Ile Leu Glu Lys Asn Arg Ala Gly Ala Arg
            420                 425                 430

Gly Thr Val Lys Leu Met Phe Gln Lys Glu Tyr Asn Lys Phe Ser Ser
        435                 440                 445

Ile Ala Gln Phe Glu Glu Arg
    450                 455
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Fragments of the above polypeptides or proteins are also encompassed by the present invention.

Suitable fragments can be produced by several means. In the first, subclones of the gene encoding the protein of the present invention are produced by conventional molecular genetic manipulation by subcloning gene fragments. The subclones then are expressed *in vitro* or *in vivo* in bacterial cells to yield a smaller

protein or peptide that can be tested for activity according to the procedures described below.

As an alternative, fragments of replication proteins can be produced by digestion of a full-length replication protein with proteolytic enzymes like chymotrypsin or *Staphylococcus* proteinase A, or trypsin. Different proteolytic enzymes are likely to cleave replication proteins at different sites based on the amino acid sequence of the protein. Some of the fragments that result from proteolysis may be active and can be tested for activity as described below.

In another approach, based on knowledge of the primary structure of the protein, fragments of a replication protein gene may be synthesized by using the PCR technique together with specific sets of primers chosen to represent particular portions of the protein. These then would be cloned into an appropriate vector for increased expression of a truncated peptide or protein.

Chemical synthesis can also be used to make suitable fragments. Such a synthesis is carried out using known amino acid sequences of replication proteins being produced. Alternatively, subjecting a full length replication protein to high temperatures and pressures will produce fragments. These fragments can then be separated by conventional procedures (e.g., chromatography, SDS-PAGE).

Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the properties, secondary structure, and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which cotranslationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification, or identification of the polypeptide.

Suitable DNA molecules are those that hybridize to a DNA molecule comprising a nucleotide sequence of at least about 20, more preferably at least about 30 to about 50, continuous bases of either SEQ. ID. Nos. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, or 33 under stringent conditions such as those characterized by a hybridization buffer comprising 0.9M sodium citrate ("SSC") buffer at a temperature of about 37°C and remaining bound when subject to washing the SSC buffer at a temperature of about 37°C; and preferably in a hybridization buffer comprising 20% formamide in 0.9M SSC buffer at a temperature of about 42°C and

remaining bound when subject to washing at about 42°C with 0.2x SSC buffer. Stringency conditions can be further varied by modifying the temperature and/or salt content of the buffer, or by modifying the length of the hybridization probe.

The proteins or polypeptides of the present invention are preferably produced in purified form (preferably at least 80%, more preferably 90%, pure) by conventional techniques. Typically, the proteins or polypeptides of the present invention is secreted into the growth medium of recombinant host cells. Alternatively, the proteins or polypeptides of the present invention are produced but not secreted into growth medium. In such cases, to isolate the protein, the host cell (e.g., *E. coli*) carrying a recombinant plasmid is propagated, lysed by sonication, heat, or chemical treatment, and the homogenate is centrifuged to remove bacterial debris. The supernatant is then subjected to purification procedures such as ammonium sulfate precipitation, gel filtration, ion exchange chromatography, FPLC, and HPLC.

The DNA molecule encoding replication polypeptides or proteins derived from Gram positive bacteria can be incorporated in cells using conventional recombinant DNA technology. Generally, this involved inserting the DNA molecule into an expression system to which the DNA molecule is heterologous (i.e. not normally present). The heterologous DNA molecule is inserted into the expression system or vector in proper sense orientation and correct reading frame. The vector contains the necessary elements for the transcription and translation of the inserted protein-coding sequences.

U.S. Patent No. 4,237,224 to Cohen and Boyer, which is hereby incorporated by reference, describes the production of expression systems in the form of recombinant plasmids using restriction enzyme cleavage and ligation with DNA ligase. These recombinant plasmids are then introduced by means of transformation and replicated in unicellular cultures including procaryotic organisms and eucaryotic cells grown in tissue culture.

Recombinant genes may also be introduced into viruses, such as vaccina virus. Recombinant viruses can be generated by transfection of plasmids into cells infected with virus.

Suitable vectors include, but are not limited to, the following viral vectors such as lambda vector system gt11, gt WES.tB, Charon 4, and plasmid vectors such as pBR322, pBR325, pACYC177, pACYC1084, pUC8, pUC9, pUC18, pUC19,

pLG339, pR290, pKC37, pKC101, SV 40, pBluescript II SK +/- or KS +/- (see "Stratagene Cloning Systems" Catalog (1993) from Stratagene, La Jolla, Calif, which is hereby incorporated by reference), pQE, pIH821, pGEX, pET series (see F.W. Studier et al., "Use of T7 RNA Polymerase to Direct Expression of Cloned Genes," Gene Expression Technology vol. 185 (1990), which is hereby incorporated by reference), and any derivatives thereof. Recombinant molecules can be introduced into cells via transformation, particularly transduction, conjugation, mobilization, or electroporation. The DNA sequences are cloned into the vector using standard cloning procedures in the art, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Laboratory, Cold Springs Harbor, New York (1989), which is hereby incorporated by reference.

A variety of host-vector systems may be utilized to express the protein-encoding sequence(s). Primarily, the vector system must be compatible with the host cell used. Host-vector systems include but are not limited to the following: bacteria transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA; microorganisms such as yeast containing yeast vectors; mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); and plant cells infected by bacteria. The expression elements of these vectors vary in their strength and specificities. Depending upon the host-vector system utilized, any one of a number of suitable transcription and translation elements can be used.

Different genetic signals and processing events control many levels of gene expression (e.g., DNA transcription and messenger RNA (mRNA) translation).

Transcription of DNA is dependent upon the presence of a promotor which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eucaryotic promoters differ from those of procaryotic promoters. Furthermore, eucaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a procaryotic system, and, further procaryotic promoters are not recognized and do not function in eucaryotic cells.

Similarly, translation of mRNA in procaryotes depends upon the presence of the proper procaryotic signals which differ from those of eukaryotes. Efficient translation of mRNA in procaryotes requires a ribosome binding site called

the Shine-Dalgarno ("SD") sequence on the mRNA. This sequence is a short nucleotide sequence of mRNA that is located before the same codon, usually AUG, which encodes the amino-terminal methionine of the protein. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology, 68:473 (1979), which is hereby incorporated by reference.

Promoters vary in their "strength" (i.e. their ability to promote transcription). For the purposes of expressing a cloned gene, it is desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of the gene. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when cloning in *E. coli*, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promotor, *trp* promotor, *rec*A promotor, ribosomal RNA promotor, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lac*UV5, *omp*F, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lac*UV5 (*tac*) promotor or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene.

Bacterial host cell strains and expression vectors may be chosen which inhibit the action of the promotor unless specifically induced. In certain operations, the addition of specific inducers is necessary for efficient transcription of the inserted DNA. For example, the *lac* operon is induced by the addition of lactose or IPTG (isopropylthio-beta-D-galactoside). A variety of other operons, such as *trp*, *pro*, etc., are under different controls. Additionally, the cell may carry the gene for a heterologous RNA polymerase such as from phage T7. Thus, a promoter specific for T7 RNA polymerase is used. The T7 RNA polymerase may be under inducible control.

Specific initiation signals are also required for efficient gene transcription and translation in procaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector,

which contains a promotor, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. Thus, an SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the *N* gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used.

Once the isolated DNA molecule encoding a replication polypeptide or protein has been cloned into an expression system, it is ready to be incorporated into a host cell. Such incorporation can be carried out by the various forms of transformation noted above, depending upon the vector/host cell system. Suitable host cells include, but are not limited to, bacteria, viruses, yeast, mammalian cells, insects, plants, and the like.

The invention provides efficient methods of identifying pharmacological agents or lead compounds for agents active at the level of a replication protein function, particularly DNA replication. Generally, these screening methods involve assaying for compounds which interfere with the replication activity. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development. Target therapeutic indications are limited only in that the target cellular function be subject to modulation, usually inhibition, by disruption of a replication activity or the formation of a complex comprising a replication protein and one or more natural intracellular binding targets. Target indications may include arresting cell growth or causing cell death resulting in recovery from the bacterial infection in animal studies.

A wide variety of assays for activity and binding agents are provided, including DNA synthesis, ATPase, clamp loading onto DNA, protein-protein binding assays, immunoassays, cell based assays, etc. The replication protein compositions, used to identify pharmacological agents, are in isolated, partially pure or pure form

and are typically recombinantly produced. The replication protein may be part of a fusion product with another peptide or polypeptide (e.g., a polypeptide that is capable of providing or enhancing protein-protein binding, stability under assay conditions (e.g., a tag for detection or anchoring), etc.). The assay mixtures comprise a natural intracellular replication protein binding target such as DNA, another protein, NTP, or dNTP. For binding assays, while native binding targets may be used, it is frequently preferred to use portions (e.g., peptides, nucleic acid fragments) thereof so long as the portion provides binding affinity and avidity to the subject replication protein conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Generally, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control (i.e., at zero concentration or below the limits of assay detection). Additional controls are often present such as a positive control, a dose response curve, use of known inhibitors, use of control heterologous proteins, etc. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably they are small organic compounds and are obtained from a wide variety of sources, including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins (e.g., albumin, detergents, etc.), which may be used to facilitate optimal binding and/or reduce nonspecific or background interactions, etc. Also reagents that otherwise improve the efficiency of the assay (e.g., protease inhibitors, nuclease inhibitors, antimicrobial agents, etc.) may be used.

The invention provides replication protein specific assays and the binding agents including natural intracellular binding targets such as other replication proteins, etc., and methods of identifying and making such agents, and their use in a variety of diagnostic and therapeutic applications, especially where disease is associated with excessive cell growth. Novel replication protein-specific binding agents include replication protein-specific antibodies and other natural intracellular binding agents identified with assays such as one- and two-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries, etc.

Generally, replication protein-specificity of the binding agent is shown by binding equilibrium constants. Such agents are capable of selectively binding a

replication protein (i.e., with an equilibrium constant at least about 10^7 M^{-1}, preferably, at least about 10^8 M^{-1}, more preferably, at least about 10^9 M^{-1}). A wide variety of cell-based and cell-free assays may be used to demonstrate replication protein-specific activity, binding, gel shift assays, immunoassays, etc.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the replication protein specifically binds the cellular binding target, portion, or analog. The mixture of components can be added in any order that provides for the requisite bindings. Incubations may be performed at any temperature which facilitates optimal binding, typically between 4°C and 40°C, more commonly between 15°C and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours.

After incubation, the presence or absence of activity or specific binding between the replication protein and one or more binding targets is detected by any convenient way. For cell-free activity and binding type assays, a separation step may be used to separate the activity product or the bound from unbound components. Separation may be effected by precipitation (e.g., immunoprecipitation), immobilization (e.g., on a solid substrate such as a microtiter plate), etc., followed by washing. Many assays that do not require separation are also possible such as use of europium conjugation in proximity assays or a detection system that is dependent on a product or loss of substrate.

Detection may be effected in any convenient way. For cell-free activity and binding assays, one of the components usually comprises or is coupled to a label. A wide variety of labels may be employed – essentially any label that provides for detection of DNA product, loss of DNA substrate, conversion of a nucleotide substrate, or bound protein is useful. The label may provide for direct detection such as radioactivity, fluorescence, luminescence, optical, or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. The label may be appended to the protein (e.g., a phosphate group comprising a radioactive isotope of phosphorous), or incorporated into the DNA substrate or the protein structure (e.g., a methionine residue comprising a radioactive isotope of sulfur.) A variety of methods may be used to detect the label depending on the nature of the label and other assay

components. For example, the label may be detected bound to the solid substrate, or a portion of the bound complex containing the label may be separated from the solid substrate, and thereafter the label detected. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfer, fluorescence emission, etc. or indirectly detected with antibody conjugates, etc. For example, in the case of radioactive labels, emissions may be detected directly (e.g., with particle counters) or indirectly (e.g., with scintillation cocktails and counters).

The present invention identifies the set of proteins that together result in a three component polymerase from bacteria that are distantly related to *E. coli*, such as Gram positive bacteria. Specifically, these bacteria lack several genes that *E. coli* DNA polymerase III has, such as *holD, holD* or *holE*. Further, *dnaX* is believed to encode only one protein, tau. Also, *holA* is quite divergent in homology suggesting it may function in another process in these organisms. Gram positive cells even have replication genes that *E. coli* does not, implying that they may not utilize the replication strategies exemplified by *E. coli*.

The present invention identifies genes and proteins that form a three component polymerase in Gram positive organisms, such as *S. pyogenes* and *S. aureus*. In *S. pyogenes* and *S. aureus*, the polymerase α–large, functions with a β clamp and a clamp loader component of τδδ'. They display high speed and processivity in synthesis of ssDNA coated with SSB and primed with a DNA oligonucleotide.

This invention also expresses and purifies a protein from a Gram positive bacteria that is homologous to the *E. coli* beta subunit. The invention demonstrates that it behaves like a circular protein. Further, this invention shows that a beta subunit from a Gram positive bacteria is functional with both Pol III-L (α-large) from a Gram positive bacteria and with DNA polymerase III from a Gram negative bacteria. This result can be explained by an interaction between the clamp and the polymerase that has been conserved during the evolutionary divergence of Gram positive and Gram negative cells. A chemical inhibitor that would disrupt this interaction would be predicted to have a broad spectrum of antibiotic activity, shutting down replication in gram negative and gram positive cells alike. This assay, and others based on this interaction, can be devised to screen chemicals for such inhibition. Further, since all the proteins in this assay are highly overexpressed

through recombinant techniques, sufficient quantities of the protein reagents can be obtained for screening hundreds of thousands of compounds.

This invention also shows that the DnaE polymerase (α-small), encoded by the *dnaE* gene, functions with the beta clamp and τδδ′ complex. The speed of DnaE is not significantly increased by τδδ′ and β, but the processivity of DnaE is greatly increased by τδδ′ and β. Hence, the DnaE polymerase, coupled with its β clamp on DNA (loaded by τδδ′) may also be an important target for a candidate pharmaceutical drug.

The present invention provides methods by which replication proteins from a Gram positive bacteria are used to discover new pharmaceutical agents. The function of replication proteins is quantified in the presence of different chemical compounds. A chemical compound that inhibits the function is a candidate antibiotic. Some replication proteins from a Gram positive bacteria and from a Gram negative bacteria can be interchanged for one another. Hence, they can function as mixtures. Reactions that assay for the function of enzyme mixtures consisting of proteins from Gram positive bacteria and from Gram negative bacteria can also be used to discover drugs. Suitable *E. coli* replication proteins are the subunits of its Pol III holoenzyme which are described in U.S. Patent Nos. 5,583,026 and 5,668,004 to O'Donnell, which are hereby incorporated by reference.

The methods described herein to obtain genes, and the assays demonstrating activity behavior of *S. pyogenes* and *S. aureus* replication proteins are likely to generalize to all members of the *Streptococcus* and *Staphylococcus* genuses, as well as to all Gram positive bacteria. Such assays are also likely to generalize to other cells besides Gram positive bacteria which also share features in common with *S. pyogenes* and *S. aureus* that are different from *E. coli* (i.e., lacking *holC, holD,* or *holE*; having a *dnaX* gene encoding a single protein; or having a weak homology to *holA* encoding delta).

The present invention describes a method of identifying compounds which inhibit the activity of a polymerase product of *polC* or *dnaE*. This method is carried out by forming a reaction mixture that includes a primed DNA molecule, a polymerase product of *polC* or *dnaE*, a candidate compound, a dNTP, and optionally either a beta subunit, a tau complex, or both the beta subunit and the tau complex, wherein at least one of the polymerase product of *polC* or *dnaE*, the beta subunit, the

tau complex, or a subunit or combination of subunits thereof is derived from a Eubacteria other than *Escherichia coli*; subjecting the reaction mixture to conditions effective to achieve nucleic acid polymerization in the absence of the candidate compound; analyzing the reaction mixture for the presence or absence of nucleic acid polymerization extension products; and identifying the candidate compound in the reaction mixture where there is an absence of nucleic acid polymerization extension products. Preferably, the polymerase product of *polC* or *dnaE*, the beta subunit, the tau complex, or the subunit or combination of subunits thereof is derived from a Gram positive bacterium, more preferably a *Streptococcus* bacterium such as *S. pyogenes* or a *Staphylococcus* bacterium such as *S. aureus*.

The present invention describes a method to identify chemicals that inhibit the activity of the three component polymerase. This method involves contacting primed DNA with the DNA polymerase in the presence of the candidate pharmaceutical, and dNTPs (or modified dNTPs) to form a reaction mixture. The reaction mixture is subjected to conditions effective to achieve nucleic acid polymerization in the absence of the candidate pharmaceutical and the presence or absence of the extension product in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of product.

The present invention describes a method to identify candidate pharmaceuticals that inhibit the activity of a clamp loader complex and a beta subunit in stimulating the DNA polymerase. The method includes contacting a primed DNA (which may be coated with SSB) with a DNA polymerase, a beta subunit, and a tau complex (or subunit or subassembly of the tau complex) in the presence of the candidate pharmaceutical, and dNTPs (or modified dNTPs) to form a reaction mixture. The reaction mixture is subjected to conditions which, in the absence of the candidate pharmaceutical, would effect nucleic acid polymerization and the presence or absence of the extension product in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of product. The DNA polymerase, the beta subunit, and/or the tau complex or subunit(s) thereof are derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the ability of a beta subunit and a DNA polymerase to interact physically. This method involves contacting the beta subunit with the DNA polymerase in the presence

of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions under which the DNA polymerase and the beta subunit would interact in the absence of the candidate pharmaceutical. The reaction mixture is then analyzed for interaction between the beta unit and the DNA polymerase. The candidate pharmaceutical is detected by the absence of interaction between the beta subunit and the DNA polymerase. The DNA polymerase and/or the beta subunit are derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the ability of a beta subunit and a tau complex (or a subunit or subassembly of the tau complex) to interact. This method includes contacting the beta subunit with the tau complex (or subunit or subassembly of the tau complex) in the presence of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions under which the tau complex (or the subunit or subassembly of the tau complex) and the beta subunit would interact in the absence of the candidate pharmaceutical. The reaction mixture is then analyzed for interaction between the beta subunit and the tau complex (or the subunit or subassembly of the tau complex). The candidate pharmaceutical is detected by the absence of interaction between the beta subunit and the tau complex (or the subunit or subassembly of the tau complex) . The beta subunit and/or the tau complex or subunit thereof is derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the ability of a tau complex (or a subassembly of the tau complex) to assemble a beta subunit onto a DNA molecule. This method involves contacting a circular primed DNA molecule (which may be coated with SSB) with the tau complex (or the subassembly thereof) and the beta subunit in the presence of the candidate pharmaceutical, and ATP or dATP to form a reaction mixture. The reaction mixture is subjected to conditions under which the tau complex (or subassembly) assembles the beta subunit on the DNA molecule absent the candidate pharmaceutical. The presence or absence of the beta subunit on the DNA molecule in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of the beta subunit on the DNA molecule. The beta subunit and/or the tau complex are derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the ability of a tau complex (or a subunit(s) of the tau complex) to disassemble a beta subunit from a DNA molecule. This method comprises contacting a DNA molecule onto which the beta subunit has been assembled in the presence of the candidate pharmaceutical, to form a reaction mixture. The reaction mixture is subjected to conditions under which the tau complex (or a subunit(s) or subassembly of the tau complex) disassembles the beta subunit from the DNA molecule absent the candidate pharmaceutical. The presence or absence of the beta subunit on the DNA molecule in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the presence of the beta subunit on the DNA molecule. The beta subunit and/or the tau complex are derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that disassemble a beta subunit from a DNA molecule. This method involves contacting a circular primed DNA molecule (which may be coated with SSB) upon which the beta subunit has been assembled (e.g. by action of the tau complex) with the candidate pharmaceutical. The presence or absence of the beta subunit on the DNA molecule in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of the beta subunit on the DNA molecule. The beta subunit is derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the dATP/ATP binding activity of a tau complex or a tau complex subunit (e.g. tau subunit). This method includes contacting the tau complex (or the tau complex subunit) with dATP/ATP either in the presence or absence of a DNA molecule and/or the beta subunit in the presence of the candidate pharmaceutical to form a reaction. The reaction mixture is subjected to conditions in which the tau complex (or the subunit of tau complex) interacts with dATP/ATP in the absence of the candidate pharmaceutical. The reaction is analyzed to determine if dATP/ATP is bound to the tau complex (or the subunit of tau complex) in the presence of the candidate pharmaceutical. The candidate pharmaceutical is detected by the absence of hydrolysis. The tau complex and/or the beta subunit is derived from a Gram positive bacterium.

The present invention describes a method to identify chemicals that inhibit the dATP/ATPase activity of a tau complex or a tau complex subunit (e.g., the

tau subunit). This method involves contacting the tau complex (or the tau complex subunit) with dATP/ATP either in the presence or absence of a DNA molecule and/or a beta subunit in the presence of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions in which the tau subunit (or complex) hydrolyzes dATP/ATP in the absence of the candidate pharmaceutical. The reaction is analyzed to determine if dATP/ATP was hydrolyzed. Suitable candidate pharmaceuticals are identified by the absence of hydrolysis. The tau complex and/or the beta subunit is derived from a Gram positive bacterium.

Further methods for identifying chemicals that inhibit the activity of a DNA polymerase encoded by either the *dnaE* gene, *polC* gene, or their accessory proteins (i.e., clamp loader, clamp, etc.), are as follows:

1) Contacting a primed DNA molecule with the encoded product of the *dnaE* gene or *polC* gene in the presence of the candidate pharmaceutical, and dNTPs (or modified dNTPs) to form a reaction mixture. The reaction mixture is subjected to conditions, which in the absence of the candidate pharmaceutical, affect nucleic acid polymerization and the presence or absence of the extension product in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of extension product. The protein encoded by the *dnaE* gene and *PolC* gene is derived from a Gram positive bacterium.

2) Contacting a linear primed DNA molecule with a beta subunit and the encoded product of *dnaE* or *PolC* in the presence of the candidate pharmaceutical, and dNTPs (or modified dNTPs) to form a reaction mixture. The reaction mixture is subjected to conditions, which in the absence of the candidate pharmaceutical, affect nucleic acid polymerization, and the presence or absence of the extension product in the reaction mixture is analyzed. The candidate pharmaceutical is detected by the absence of extension product. The protein encoded by the *dnaE* gene and *PolC* gene is derived from a Gram positive bacterium.

3) Contacting a circular primed DNA molecule (may be coated with SSB) with a tau complex, a beta subunit and the encoded product of a *dnaE* gene or *PolC* gene in the presence of the candidate pharmaceutical, and dNTPs (or modified dNTPs) to form a reaction mixture. The reaction mixture is subjected to conditions, which in the absence of the candidate pharmaceutical, affect nucleic acid polymerization, and the presence or absence of the extension product in the reaction

mixture is analyzed. The candidate pharmaceutical is detected by the absence of product. The protein encoded by the *dnaE* gene and *PolC* gene, the beta subunit, and/or the tau complex are derived from a Gram positive bacterium.

4) Contacting a beta subunit with the product encoded by a *dnaE* gene or *PolC* gene in the presence of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is then analyzed for interaction between the beta subunit and the product encoded by the *dnaE* gene or *PolC* gene. The candidate pharmaceutical is detected by the absence of interaction between the beta subunit and the product encoded by the *dnaE* gene or *PolC* gene. The beta subunit and/or the protein encoded by the *dnaE* gene and *PolC* gene is derived from a Gram positive bacterium.

5) The present invention discloses a method to identify chemicals that inhibit a DnaB helicase. The method includes contacting the DnaB helicase with a DNA molecule substrate that has a duplex region in the presence of a nucleoside or deoxynucleoside triphosphate energy source and a candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions that support helicase activity in the absence of the candidate pharmaceutical. The DNA duplex molecule in the reaction mixture is analyzed for whether it is converted to ssDNA. The candidate pharmaceutical is detected by the absence of conversion of the duplex DNA molecule to the ssDNA molecule. The DnaB helicase is derived from a Gram positive bacterium.

6) The present invention describes a method to identify chemicals that inhibit the nucleoside or deoxynucleoside triphosphatase activity of a DnaB helicase. The method includes contacting the DnaB helicase with a DNA molecule substrate that has a duplex region in the presence of a nucleoside or deoxynucleoside triphosphate energy source and the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions that support nucleoside or deoxynucleoside triphosphatase activity of the DnaB helicase in the absence of the candidate pharmaceutical. The candidate pharmaceutical is detected by the absence of conversion of nucleoside or deoxynucleoside triphosphate to nucleoside or deoxynucleoside diphosphate. The DnaB helicase is derived from a Gram positive bacterium.

7) The present invention describes a method to identify chemicals that inhibit a primase. The method includes contacting primase with a ssDNA molecule in the presence of a candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions that support primase activity (e.g., the presence of nucleoside or deoxynucleoside triphosphates, appropriate buffer, presence or absence of DnaB helicase) in the absence of the candidate pharmaceutical. Suitable candidate pharmaceuticals are identified by the absence of primer formation detected either directly or indirectly. The primase is derived from a Gram positive bacterium.

8) The present invention describes a method to identify chemicals that inhibit the ability of a primase and the protein encoded by a *dnaB* gene to interact. This method includes contacting the primase with the protein encoded by the *dnaB* gene in the presence of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions under which the primase and the protein encoded by the *dnaB* gene interact in the absence of the candidate pharmaceutical. The reaction mixture is then analyzed for interaction between the primase and the protein encoded by the *dnaB* gene. The candidate pharmaceutical is detected by the absence of interaction between the primase and the protein encoded by the *dnaB* gene. The primase and/or the *dnaB* gene are derived from a Gram positive bacterium.

9) The present invention describes a method to identify chemicals that inhibit the ability of a protein encoded by a *dnaB* gene to interact with a DNA molecule. This method includes contacting the protein encoded by the *dnaB* gene with the DNA molecule in the presence of the candidate pharmaceutical to form a reaction mixture. The reaction mixture is subjected to conditions under which the DNA molecule and the protein encoded by the *dnaB* gene interact in the absence of the candidate pharmaceutical. The reaction mixture is then analyzed for interaction between the protein encoded by the *dnaB* gene and the DNA molecule. The candidate pharmaceutical is detected by the absence of interaction between the DNA molecule and the protein encoded by the *dnaB* gene. The *dnaB* gene is derived from a Gram positive bacterium.

EXAMPLES

The following examples are provided to illustrate embodiments of the present invention, but they are by no means intended to limit its scope.

Example 1 - Materials

Labeled deoxy- and ribonucleoside triphosphates were from Dupont-New England Nuclear; unlabelled deoxy- and ribonucleoside triphosphates were from Pharmacia-LKB; *E. coli* replication proteins were purified as described, alpha, epsilon, gamma, and tau (Studwell et al., "Processive Replication is Contingent on the Exonuclease Subunit of DNA Polymerase III Holoenzyme," J. Biol. Chem., 265:1171-1178 (1990), which is hereby incorporated by reference), beta (Kong et al., "Three Dimensional Structure of the Beta Subunit of *Escherichia coli* DNA Polymerase III Holoenzyme: A Sliding DNA Clamp," Cell, 69:425-437 (1992), which is hereby incorporated by reference), delta and delta prime (Dong et al., "DNA Polymerase III Accessory Proteins. I. *HolA* and *holB* Encoding δ and δ'," J. Biol. Chem., 268:11758-11765 (1993), which is hereby incorporated by reference), chi and psi (Xiao et al., "DNA Polymerase III Accessory Proteins. III. *HolC* and *holD* Encoding chi and psi," J. Biol. Chem., 268:11773-11778 (1993), which is hereby incorporated by reference), theta (Studwell-Vaughan et al., "DNA Polymerase III Accessory Proteins. V. Theta Encoded by *holE*," J. Biol. Chem., 268:11785-11791 (1993), which is hereby incorporated by reference), and SSB (Weiner et al., "The Deoxyribonucleic Acid Unwinding Protein of *Escherichia coli*," J. Biol. Chem., 250:1972-1980 (1975), which is hereby incorporated by reference). *E. coli* Pol III core and clamp loader complex (composed of subunits gamma, delta, delta prime, chi, and psi) were reconstituted as described in Onrust et al., "Assembly of a Chromosomal Replication Machine: Two DNA Polymerases, a Clamp Loader and Sliding Clamps in One Holoenzyme Particle. I. Organization of the Clamp Loader," J. Biol. Chem., 270:13348-13357 (1995), which is hereby incorporated by reference. Pol III* was reconstituted and purified as described in Onrust et al., "Assembly of a Chromosomal Replication Machine: Two DNA Polymerases, a Clamp Loader and Sliding Clamps in One Holoenzyme Particle. III. Interface Between Two Polymerases and the Clamp

Loader," J. Biol. Chem., 270:13366-13377 (1995), which is hereby incorporated by reference. Protein concentrations were quantitated by the Protein Assay (Bio-Rad) method using bovine serum albumin (BSA) as a standard. DNA oligonucleotides were synthesized by Oligos etc. Calf thymus DNA was from Sigma. Buffer A is 20 mM Tris-HCl (pH=7.5), 0.5 mM EDTA, 2 mM DTT, and 20% glycerol. Replication buffer is 20 mM Tris-Cl (pH 7.5), 8 mM $MgCl_2$, 5 mM DTT, 0.5 mM EDTA, 40 μg/ml BSA, 4% glycerol, 0.5 mM ATP, 3 mM each dCTP, dGTP, dATP, and 20 μM [α-^{32}P]dTTP. P-cell buffer is 50 mM potassium phosphate (pH 7.6), 5 mM DTT, 0.3 mM EDTA, 20% glycerol. T.E. buffer is 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. Cell lysis buffer is 50 mM Tris-HCl (pH 8.0) 10 % sucrose, 1 M NaCl, 0.3 mM spermidine.

Example 2 - Calf Thymus DNA Replication Assays

These assays were used in the purification of DNA polymerases from *S. aureus* cell extracts. Assays contained 2.5 μg activated calf thymus DNA in a final volume of 25 μl replication buffer. An aliquot of the fraction to be assayed was added to the assay mixture on ice followed by incubation at 37°C for 5 min. DNA synthesis was quantitated using DE81 paper as described in Rowen et al., "Primase, the DnaG Protein of *Escherichia coli*. An Enzyme Which Starts DNA Chains," J. Biol. Chem., 253:758-764 (1979), which is hereby incorporated by reference.

Example 3 - PolydA-oligodT Replication Assays

PolydA-oligodT was prepared as follows. PolydA of average length 4500 nucleotides was purchased from SuperTecs. OligodT35 was synthesized by Oligos etc. 145 ul of 5.2 mM (as nucleotide) polydA and 22 μl of 1.75 mM (as nucleotide) oligodT were mixed in a final volume of 2100 μl T.E. buffer (ratio as nucleotide was 21:1 polydA to oligodT). The mixture was heated to boiling in a 1 ml eppendorf tube, then removed and allowed to cool to room temperature. Assays were performed in a final volume of 25 μl 20 mM Tris-Cl (pH 7.5), 8 mM $MgCl_2$, 5 mM DTT, 0.5 mM EDTA, 40 μg/ml BSA, 4% glycerol, containing 20 μM [α-^{32}P]dTTP

and 0.36 μg polydA-oligodT. Proteins were added to the reaction on ice, then shifted to 37°C for 5 min. DNA synthesis was quantitated using DE81 paper as described in Rowen et al., "Primase, the DnaG Protein of *Escherichia coli*. An Enzyme Which Starts DNA Chains," J. Biol. Chem., 253:758-764 (1979), which is hereby incorporated by reference.

Example 4 - Singly Primed M13mp18 ssDNA Replication Assays

M13mp18 was phenol extracted from phage and purified by two successive bandings (one downward and one upward) in cesium chloride gradients. M13mp18 ssDNA was singly primed with a DNA 30mer (map position 6817-6846) as described in Studwell et al. "Processive Replication is Contingent on the Exonuclease Subunit of DNA Polymerase III Holoenzyme," J. Biol. Chem., 265:1171-1178 (1990), which is hereby incorporated by reference. Replication assays contained 72 ng of singly primed M13mp18 ssDNA in a final volume of 25 μl of replication buffer. Other proteins added to the assay, and their amounts, are indicated in the Brief Description of the Drawings. Reactions were incubated for 5 min. at 37°C and then were quenched upon adding an equal volume of 1% SDS and 40 mM EDTA. DNA synthesis was quantitated using DE81 paper as described in Rowen et al., "Primase, the DnaG Protein of *Escherichia coli*. An Enzyme Which Starts DNA Chains," J. Biol. Chem., 253:758-764 (1979), which is hereby incorporated by reference, and product analysis was performed in a 0.8% native agarose gel followed by autoradiography.

Example 5 - Genomic *Staphylococcus aureus* DNA

Two strains of *S. aureus* were used. For PCR of the first fragment of the *dnaX* gene sequence, the strain was ATCC 25923. For all other work the strain was strain 4220 (a gift of Dr. Pat Schlievert, University of Minnisota). This strain lacks a gene needed for producing toxic shock (Kreiswirth et al., "The Toxic Shock Syndrome Exotoxin Structural Gene is Not Detectably Transmitted by a Prophage," Nature, 305:709-712 (1996) and Balan et al., "Autocrine Regulation of Toxin Synthesis by *Staphylococcus aureus*," Proc. Natl. Acad. Sci. USA, 92:1619-1623

(1995), which are hereby incorporated by reference). *S. aureus* cells were grown overnight at 37°C in LB containing 0.5% glucose. Cells were collected by centrifugation (24 g wet weight). Cells were resuspended in 80 ml solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCL (pH 8.0)). SDS and NaOH were then added to 1% and 0.2 N, respectively, followed by incubation at 65°C for 30 min. to lyse the cells. 68.5 ml of 3 M sodium acetate (pH 5.0) was added followed by centrifugation at 12,000 rpm for 30 min. The supernatant was discarded and the pellet was washed twice with 50 ml of 6M urea, 10 mM Tris-HCL (pH 7.5), 1 mM EDTA using a dounce homogenizer. After each wash, the resuspended pellet was collected by centrifugation (12,000 rpm for 20 min.). After the second wash, the pellet was resuspended in 50 ml 10 mM T.E. buffer using a dounce homogenizer and then incubated for 30 min. at 65°C. The solution was centrifuged at 12,000 rpm for 20 min., and the viscous supernatant was collected. 43.46 g $CsCl_2$ was added to the 50 ml of supernatant (density between 1.395-1.398) and poured into two 35 ml quick seal ultracentrifuge tubes (tubes were completely filled using the same density of $CsCl_2$ in T.E.). To each tube was added 0.5 ml of a 10 mg/ml stock of ethidium bromide. Tubes were spun at 55,000 rpm for 18 h at 18°C in a Sorvall TV860 rotor. The band of genomic DNA was extracted using a syringe and needle. Ethidium bromide was removed using two butanol extractions and then dialyzed against 4 l of T.E. at pH 8.0 overnight. The DNA was recovered by ethanol precipitation and then resuspended in T.E. buffer (1.7 mg total) and stored at -20°C.

Example 6 - Cloning and Purification of *S. aureus* Pol III-L

To further characterize the mechanism of DNA replication in *S. aureus*, large amounts of its replication proteins were produced through use of the genes. The *polC* gene encoding *S. aureus* Pol III-L (alpha-large) subunit has been sequenced and expressed in *E. coli* (Pacitti et al., "Characterization and Overexpression of the Gene Encoding *Staphylococcus aureus* DNA Polymerase III," Gene, 165:51-56 (1995), which is hereby incorporated by reference). The previous work utilized a pBS[KS] vector for expression in which the *E. coli* RNA polymerase is used for gene transcription. In the earlier study, the *S. aureus polC* gene was precisely cloned at the 5' end encoding the N-terminus, but the amount of the gene

that remained past the 3' end was not disclosed and the procedure for subcloning the gene into the expression vector was only briefly summarized. Furthermore, the previous study does not show the level of expression of the *S. aureus* Pol III-L, nor the amount of *S. aureus* Pol III-L that is obtained from the induced cells. Since the previously published procedure could not be repeated and the efficiency of the expression vector could not be assessed, another strategy outlined below had to be developed.

The isolated *polC* gene was cloned into a vector that utilizes T7 RNA polymerase for transcription as this process generally expresses a large amount of protein. Hence, the *S. aureus polC* gene was cloned precisely into the start codon at the NdeI site downstream of the T7 promotor in a pET vector . As the *polC* gene contains an internal NdeI site, the entire gene could not be amplified and placed it into the NdeI site of a pET vector. Hence, a three step cloning strategy that yielded the desired clone was devised (Figure 1). These attempts were quite frustrating initially as no products of cloning in standard *E. coli* strains such as DH5α, a typical laboratory strain for preparation of DNA, could be obtained. Finally, a cell that was mutated in several genes affecting DNA stability was useful in obtaining the desired products of cloning.

In brief, the cloning strategy required use of another expression vector (called pET1137kDa) in which the 37 kDa subunit of human RFC, the clamp loader of the human replication system, had been cloned into the pET11 vector. The gene encoding the 37kDa subunit contains an internal NsiI site, which was needed for the precise cloning of the isolated *polC* gene. This three step strategy is shown in Figure 1. In the first step, an approximately 2.3 kb section of the 5' section of the gene (encoding the N-terminus of Pol III-L) was amplified using the polymerase chain reaction (PCR). Primers were as follows:

Upstream (SEQ. ID. No. 35)

```
ggtggtaatt gtcttgcata tgacagagc                                    29
```

Downstream (SEQ. ID. No. 36)

```
agcgattaag tggattgccg ggttgtgatg c                                 31
```

Amplification was performed using 500 ng genomic DNA, 0.5 mM EDTA, 1 μM of each primer, 1mM $MgSO_4$, 2 units vent DNA polymerase (New England Biolabs) in 100 μl of vent buffer (New England Biolabs). Forty cycles were performed using the following cycling scheme: 94°C, 1 min; 60°C, 1 min.; 72°C, 2.5 min. The product was digested with NdeI (underlined in the upstream primer) and NsiI (an internal site in the product) and the approximately 1.8 kb fragment was gel purified. A pET11 vector containing as an insert the 37 kDa subunit of human replication factor C (pET1137kDa) was digested with NdeI and NsiI and gel purified. The PCR fragment was ligated into the digested pET1137kDa vector and the ligation reaction was transformed into Epicurean coli supercompetent SURE 2 cells (Stratagene) and colonies were screened for the correct chimera (pET11PolC1) by examining minipreps for proper length and correct digestion products using NdeI and NsiI. In the second step, an approximately 2076 bp fragment containing the DNA encoding the C-terminus of Pol III-L subunit was amplified using the following sequences as primers:

Upstream (SEQ. ID. No. 37)

```
agcatcacaa cccggcaatc cacttaatcg c                    31
```

Downstream (SEQ. ID. No. 38)

```
gactacgcca tgggcattaa ataaatacc                       29
```

The amplification cycling scheme was as described above except the elongation step at 72°C was for 2 min. The product was digested with BamHI (underlined in the downstream primer) and NsiI (internal to the product) and the approximately 480 bp product was gel purified and ligated into the pET11PolC1 that had been digested with NsiI/BamHI and gel purified (ligated product is pET11PolC2). To complete the expression vector, an approximately 2080 bp PCR product was amplified over the two NsiI sites internal to the gene using the following primers:

Upstream (SEQ. ID. No. 39)

```
gaagatgcat ataaacgtgc aagacctagt                      30
```

Downstream (SEQ. ID. No. 40)

```
gtctgacgca cgaattgtaa agtaagatgc atag                 34
```

The amplification cycling scheme was as described above except the 72°C elongation step was 2 min. The PCR product, and the pET11PolC2 vector, were digested with NsiI and gel purified. The ligation mixture was transformed as described above and colonies were screened for the correct chimera (pET11PolC).

To express Pol III-L polymerase, the pET11PolC plasmid was transformed into *E. coli* strain BL21(DE3). 24 L of *E. coli* BL21(DE3)pET11PolC were grown in LB media containing 50 μg/ml ampicillin at 37°C to an OD of 0.7 and then the temperature was lowered to 15°C. Cells were then induced for Pol III-L expression upon addition of 1 mM IPTG to produce the T7 RNA polymerase needed to transcribe *polC*. This step was followed by further incubation at 15°C for 18 h. Expression of *S. aureus* Pol III-L polymerase was so high that it could easily be visualized by Coomassie staining of a SDS polyacrylamide gel of whole cells (Figure 2A). The expressed protein migrated in the SDS polyacrylamide gel in a position expected for a 165 kDa polypeptide. In this procedure, it is important that cells are induced at 15°C, as induction at 37°C produces a truncated version of Pol III-L polymerase, of approximately 130 kDa.

Cells were collected by centrifugation at 5°C. Cells (12 g wet weight) were stored at -70°C. The following steps were performed at 4°C. Cells were thawed and lysed in cell lysis buffer as described (final volume = 50 ml) and were passed through a French Press (Amico) at a minimum of 20,000 psi. PMSF (2 mM) was added to the lysate as the lysate was collected from the French Press. DNA was removed and the lysate was clarified by centrifugation. The supernatent was dialyzed for 1 h against Buffer A containing 50 mM NaCl. The final conductivity was equivalent to 190 mM NaCl. Supernatent (24 ml, 208 mg) was diluted to 50 ml using Buffer A to bring the conductivity to 96 mM $MgCl_2$, and then was loaded onto an 8 ml MonoQ column equilibrated in Buffer A containing 50 mM NaCl. The column was eluted with a 160 ml linear gradient of Buffer A from 50 mM NaCl to 500 mM NaCl. Seventy five fractions (1.3 ml each) were collected (Figure 2B). Aliquots were analyzed for their ability to synthesize DNA, and 20 μl of each fraction was analyzed by Coomassie staining of an SDS polyacrylamide gel. Based on the DNA synthetic capability, and the correct size band in the gel, fractions 56-65 containing Pol III-L polymerase were pooled (22 ml, 31 mg). The pooled fractions were dialyzed

overnight at 4°C against 50 mM phosphate (pH 7.6), 5 mM DTT, 0.1 mM EDTA, 2 mM PMSF, and 20 % glycerol (P-cell buffer). The dialyzed pool was loaded onto a 4.5 ml phosphocellulose column equilibrated in P-cell buffer, and then eluted with a 25 ml linear gradient of P-cell buffer from 0 M NaCl to 0.5 M NaCl. Fractions of 1 ml were collected and analyzed in a SDS polyacrylamide gel stained with Coomassie Blue (Figure 2C). Fractions 20-36 contained the majority of the Pol III-large at a purity of greater than 90 % (5 mg).

Example 7 - *S. aureus* Pol III-L is Not Processive on its Own

The Pol III-L polymerase purifies from *B. subtilis* as a single subunit without accessory factors (Barnes et al., "Purification of DNA Polymerase III of Gram-positive Bacteria," Methods in Enzy., 262:35-42 (1995), which is hereby incorporated by reference). Hence, it seemed possible that it may be a Type I replicase (e.g., like T5 polymerase) and, thus, be capable of extending a single primer full length around a long singly primed template. To perform this experiment, a template M13mp18 ssDNA primed with a single DNA oligonucleotide either in the presence or absence of SSB was used. DNA products were analyzed in a neutral agarose gel which resolved products by size. The results showed that Pol III-L polymerase was incapable of extending the primer around the DNA (to form a completed duplex circle referred to as replicative form II ("RFII")) whether SSB was present or not. This experiment has been repeated using more enzyme and longer times, but no full length RFII products are produced. Hence, Pol III-L would appear not to follow the paradigm of the T5 system (Type I replicase) in which the polymerase is efficient in synthesis in the absence of any other protein(s).

Example 8 - Cloning and Purification of *S. aureus* Beta Subunit

The sequence of an *S. aureus* homolog of the *E. coli dnaN* gene (encoding the beta subunit) was obtained in a study in which the large recF region of DNA was sequenced (Alonso et al., "Nucleotide Sequence of the recF Gene Cluster From *Staphylococcus aureus* and Complementation Analysis in *Bacillus subtilis recF* Mutants," Mol. Gen. Genet., 246:680-686 (1995), Alonso et al., "Nucleotide

Sequence of the recF Gene Cluster From *Staphylococcus aureus* and Complementation Analysis in *Bacillus subtilis recF* Mutants," Mol. Gen. Genet., 248:635-636 (1995), which are hereby incorporated by reference). Sequence alignment of the *S. aureus* beta and *E. coli* beta show approximately 30% identity. Overall this level of homology is low and makes it uncertain that *S. aureus* beta will have the same shape and function as the *E. coli* beta subunit.

To obtain *S. aureus* beta protein, the *dnaN* gene was isolated and precisely cloned into a pET vector for expression in *E. coli*. *S. aureus* genomic DNA was used as template to amplify the homolog of the *dnaN* gene (encoding the putative beta). The upstream and downstream primers were designed to isolate the *dnaN* gene by PCR amplification from genomic DNA. Primers were:

Upstream (SEQ. ID. No. 41)

```
cgactggaag gagttttaac atatgatgga attcac                    36
```

Downstream (SEQ. ID. No. 42)

```
ttatatggat ccttagtaag ttctgattgg                           30
```

The NdeI site used for cloning into pET16b (Novagen) is underlined in the Upstream primer and the BamHI site used for cloning into pET16b is underlined in the Downstream primer. The NdeI and BamHI sites were used for directional cloning into pET16 (Figure 3). Amplification was performed using 500 ng genomic DNA, 0.5 mM dNTPs, 1 μM of each primer, 1mM $MgSO_4$, 2 units vent DNA polymerase in 100 ul of vent buffer. Forty cycles were performed using the following cycling scheme: 94°C, 1 min; 60°C, 1 min.; 72°C, 1 min. 10s. The 1167 bp product was digested with NdeI and BamHI and purified in a 0.7 % agarose gel. The pure digested fragment was ligated into the pET16b vector which had been digested with NdeI and BamHI and gel purified in a 0.7% agarose gel. Ligated products were transformed into *E. coli* competent SURE II cells (Stratagene) and colonies were screened for the correct chimera by examining minipreps for proper length and correct digestion products using NdeI and BamHI.

24 L of of BL21(DE3)pETbeta cells were grown in LB containing 50 μg/ml ampicillin at 37°C t an O.D. of 0.7, and, then, the temperature was lowered to

15°C. IPTG was added to a concentration of 2 mM and after a further 18 h at 15°C to induce expression of *S. aureus* beta (Figure 4A). It is interesting to note that the beta subunit, when induced at 37°C, was completely insoluble. However, induction of cells at 15°C provided strong expression of beta and, upon cell lysis, over 50% of the beta was present in the soluble fraction.

Cells were harvested by centrifugation (44 g wet weight) and stored at -70°C. The following steps were performed at 4°C. Cells (44 g wet weight) were thawed and resuspended in 45 ml 1X binding buffer (5 mM imidizole, 0.5 M NaCl, 20 mM Tris HCl (final pH 7.5)) using a dounce homogenizer. Cells were lysed using a French Pressure cell (Aminco) at 20,000 psi, and then 4.5 ml of 10 % polyamine P (Sigma) was added., Cell debris and DNA was removed by centrifugation at 13,000 rpm for 30 min. at 4°C. The pET16beta vector places a 20 residue leader containing 10 histidine residues at the N-terminus of beta. Hence, upon lysing the cells, the *S. aureus* beta was greatly purified by chromatography on a nickel chelate resin (Figure 4B). The supernatant (890 mg protein) was applied to a 10 ml HiTrap Chelating Separose column (Pharmacia-LKB) equilibrated in binding buffer. The column was washed with binding buffer, then eluted with a 100 ml linear gradient of 60 mM imidazole to 1 M imidazole in binding buffer. Fractions of 1.35 ml were collected. Fractions were analyzed for the presence of beta in an SDS polyacrylamide gel stained with Coomassie Blue. Fractions 28-52, containing most of the beta subunit, were pooled (35 ml, 82 mg). Remaining contaminating protein was removed by chromatography on MonoQ. The *S. aureus* beta becomes insoluble as the ionic strength is lowered and, thus, the pool of beta was dialyzed overnight against Buffer A containing 400 mM NaCl. The dialyzed pool became slightly turbid indicating it was at its solubility limit at these concentrations of protein and NaCl. The insoluble material was removed by centrifugation (64 mg remaining) and, then, diluted 2-fold with Buffer A to bring the conductivity to 256. The protein was then applied to an 8 ml MonoQ column equilibrated in Buffer A plus 250 mM NaCl and then eluted with a 100 ml linear gradient of Buffer A from 0.25M NaCl to 0.75 M NaCl; fractions of 1.25 ml were collected (Figure 4C).. Under these conditions, approximately 27 mg of the beta flowed through the column and the remainder eluted in fractions 1-18 (24 mg).

Example 9 - The *S. aureus* Beta Subunit Protein Stimulates *S. aureus* Pol III-L and *E. coli* Core

The experiment of Figure 5A, tests the ability of *S. aureus* beta to stimulate *S. aureus* Pol III-L on a linear polydA-oligodT template. Reactions are also performed with *E. coli* beta and Pol III core. The linear template was polydA of average length of 4500 nucleotides primed with a 30mer oligonucleotide of T residues. The first two lanes show the activity of Pol III-L either without (lane 1) or with *S. aureus* beta (lane 2). The result shows that the *S. aureus* beta stimulates Pol III-L approximately 5-6 fold. Lanes 5 and 6 show the corresponding experiment using *E. coli* core with (lane 6) or without (lane 5) *E. coli* beta. The core is stimulated over 10-fold by the *E. coli* beta subunit under the conditions used.

Although Gram positive and Gram negative cells diverged from one another long ago and components of one polymerase machinery would not be expected to be interchangable, it was decided to test the activity of the *S. aureus* beta with *E. coli* Pol III core. Lanes 3 and 4 shows that the *S. aureus* beta also stimulates *E. coli* core about 5-fold. This result can be explained by an interaction between the clamp and the polymerase that has been conserved during the evolutionary divergence of gram positive and gram negative cells. A chemical inhibitor that would disrupt this interaction would be predicted to have a broad spectrum of antibiotic activity, shutting down replication in Gram negative and Gram positive cells alike. This assay, and others based on this interaction, can be devised to screen chemicals for such inhibition. Further, since all the proteins in this assay are highly overexpressed through recombinant techniques, sufficient quantities of the protein reagents can be obtained for screening hundreds of thousands of compounds.

In summary, the results show that *S. aureus* beta, produced in *E. coli*, is indeed an active protein (i.e., it stimulates polymerase activity). Furthermore, the results shows that Pol III-L functions with a second protein (i.e., *S. aureus* beta). Before this experiment, there was no assurance that Pol III-L, which is significantly different in structure from *E. coli* alpha, would function with another protein. For example, unlike *E. coli* alpha, which copurifies with several accessory proteins, Pol III-L purified from *B. subtilis* as a single protein with no other subunits attached (Barnes et al., "Purification of DNA Polymerase III of Gram-positive Bacteria," Methods in Enzy., 262:35-42 (1995), which is hereby incorporated by reference).

Finally, if one were to assume that *S. aureus* beta would function with a polymerase, the logical candidate would have been the product of the *dnaE* gene (alpha-small) instead of *polC* (Pol III-L) since the *dnaE* product is more homologous to *E. coli* alpha subunit than Pol III-L.

Example 10 - The *S. aureus* Beta Subunit Behaves as a Circular Sliding Clamp

The ability of *S. aureus* beta to stimulate Pol III-L could be explained by formation of a 2-protein complex between Pol III-L and beta to form a processive replicase similar to the Type II class (e.g., T7 type). Alternatively, the *S. aureus* replicase is organized as the Type III replicase which operates with a circular sliding clamp and a clamp loader. In this case, the *S. aureus* beta would be a circular protein and would require a clamp loading apparatus to load it onto DNA. The ability of the beta subunit to stimulate Pol III-L in Figure 5A could be explained by the fact that the polydA-oligodT template is a linear DNA and a circular protein could thread itself onto the DNA over an end. Such "end threading" has been observed with PCNA and explains its ability to stimulate DNA polymerase delta in the absence of the RFC clamp loader (Burgers et al., "ATP-Independent Loading of the Proliferating Cell Nuclear Antigen Requires DNA Ends," J. Biol. Chem., 268:19923-19926 (1993), which is hereby incorporated by reference).

To distinguish between these possibilities, *S. aureus* beta was examined for ability to stimulate Pol III-L on a circular primed template. In Figure 5B, assays were performed using circular M13mp18 ssDNA coated with *E. coli* SSB and primed with a single oligonucleotide to test the activity of beta on circular DNA. Lane 1 shows the extent of DNA synthesis using Pol III-L alone. In lane 2, Pol III-L was supplemented with *S. aureus* beta. The *S. aureus* beta did not stimulate the activity of Pol III-L on this circular DNA (nor in the absence of SSB). Inability of *S. aureus* beta to stimulate Pol III-L is supported by the results of Figure 6, lane 1 that analyzes the product of Pol III-L action on the circular DNA in an agarose gel in the presence of *S. aureus* beta. In summary, these results show that *S. aureus* beta only stimulates Pol III-L on linear DNA, not circular DNA. Hence, the *S. aureus* beta subunit behaves as a circular protein.

Lane 3 shows the result of adding both *S. aureus* beta and *E. coli* gamma complex to Pol III-L. Again, no stimulation was observed (compare with lane 1). This result indicates that the functional contacts between the clamp and clamp loader were not conserved during evolution of Gram positive and Gram negative cells.

Controls for these reactions on circular DNA are shown for the *E. coli* system in Lanes 4-6. Addition of only beta to *E. coli* Pol III core did not result in stimulating the polymerase (compare lanes 4 and 5). However, when clamp loader complex was included with beta and core, a large stimulation of synthesis was observed (lane 6). In summary, stimulation of synthesis is only observed when both beta and clamp loader complex were present, consistent with inability of the circular beta ring to assemble onto circular DNA by itself.

Example 11 - Pol III-L Functions as a Pol III-Type Replicase with Beta and a Clamp Loader Complex to Become Processive

Next, it was determined whether *S. aureus* Pol III-L requires two components (a beta clamp and a clamp loader) to extend a primer full length around a circular primed template. In Figure 6, a template circular M13mp18 ssDNA primed with a single DNA oligonucleotide was used. DNA products were analyzed in a neutral agarose gel which resolves starting materials (labeled ssDNA in Figure 6) from completed duplex circles (labelled RFII for replicative form II). The first two lanes show, as demonstrated in other examples, that Pol III-L is incapable of extending the primer around the circular DNA in the presence of only *S. aureus* beta. In lane 4 of Figure 6, *E. coli* clamp loader complex (also known as gamma complex) and beta subunit were mixed with *S. aureus* Pol III-L in the assay containing singly primed M13mp18 ssDNA coated with SSB. If the beta clamp, assembled on DNA by clamp loader complex, provides processivity to *S. aureus* Pol III-L, the ssDNA circle should be converted into a fully duplex circle (RFII) which would be visible in an agarose gel analysis. The results of the experiment showed that the *E. coli* beta and clamp loader complex did indeed provide Pol III-L with ability to fully extend the primer around the circular DNA to form the RFII (lane 4). The negative control using only *E. coli* clamp loader complex and beta is shown in lane 3. For comparison, lane 6 shows the result of mixing the three components of the *E. coli* system (Pol III core, beta, and clamp loader complex). This reaction gives almost exclusively full length

RFII product. The qualitatively different product profile that Pol III-L gives in the agarose gel analysis compared to *E. coli* Pol III core with beta and clamp loader complex shows that the products observed using Pol III-L is not due to a contaminant of *E. coli* Pol III core in the *S. aureus* Pol III-L preparation (compare lanes 4 and 6).

It is generally thought that the polymerase of one system is specific for its SSB. However, these reactions are performed on ssDNA coated with the *E. coli* SSB protein. Hence, the *S. aureus* Pol III-L appears capable of utilizing *E. coli* SSB and the *E. coli* beta. It would appear that the only component that is not interchangeable between the Gram positive and Gram negative systems is the clamp loader complex.

Thus, the *S. aureus* Pol III-L functions as a Pol III type replicase with the *E. coli* beta clamp assembled onto DNA by a clamp loader complex.

Example 12 - Purification of Two DNA Polymerase III-Type Enzymes From *S. aureus* Cells

The MonoQ resin by Pharmacia has very high resolution which would resolve the three DNA polymerases of *S. aureus*. Hence, *S. aureus* cells were lysed, DNA was removed from the lysate, and the clarified lysate was applied onto a MonoQ column. The details of this procedure are: 300 L of *S. aureus* (strain 4220, a gift of Dr. Pat Schlievert, University of Minnisota) was grown in 2X LB media at 37°C to an O.D. of approximately 1.5 and then were collected by centrifugation. Approximately 2 kg of wet cell paste was obtained and stored at -70°C. 122 g of cell paste was thawed and resuspended in 192 ml of cell lysis buffer followed by passage through a French Press cell (Aminco) at 40,000 psi. The resultant lysate was clarified by high speed centrifugation (1.3 g protein in 120 ml). A 20 ml aliquot of the supernatant was dialyzed 2 h against 2 L of buffer A containing 50 mM NaCl. The dialyzed material (148 mg, conductivity = 101 mM NaCl) was diluted 2-fold with Buffer A containing 50 mM NaCl and then loaded onto an 8 ml MonoQ column equilibrated in Buffer A containing 50 mM NaCl. The column was washed with Buffer A containing 50 mM NaCl, and then eluted with a 160 ml linear gradient of 0.05 M NaCl to 0.5 M NaCl in Buffer A. Fractions of 2.5 ml (64 total) were collected, followed by analysis in an SDS polyacrylamide gel for their replication activity in assays using calf thymus DNA.

Three peaks of DNA polymerase activity were identified (Figure 7). Previous studies of cell extracts prepared from the Gram positive organism *Bacillus subtilis* identified only two peaks of activity off a DEAE column (similar charged resin to MonoQ). The first peak was Pol II, and the second peak was a combination of DNA polymerases I and III. The DNA polymerases I and III were then separated on a subsequent phosphocellulose column. The middle peak in Figure 7 is much larger than the other two peaks and, thus, it was decided to chromatograph this peak on a phosphocellulose column. The second peak of DNA synthetic activity was pooled (fractions 37-43; 28 mg in 14 ml) and dialyzed against 1.5 L P-cell buffer for 2.5 h. Then, the sample (ionic strength equal to 99 mM NaCl) was applied to a 5 ml phosphocellulose column equilibrated in P-cell buffer. After washing the column in 10 ml P-cell buffer, the column was eluted with a 60 ml gradient of 0 - 0.5 M NaCl in P-cell buffer. Seventy fractions were collected and then analyzed for DNA synthesis using calf thymus DNA as template. This column resolved the polymerase activity into two distinct peaks (Figure 7B).

Hence, there appear to be four DNA polymerases in *Staphylococcus aureus*. They were designated here as peak 1 (first peak off MonoQ), peak 2 (first peak off phosphocellulose), peak 3 (second peak of phosphocellulose), and peak 4 (last peak off Mono Q) (see Figure 7). Peak 4 was presumably Pol III-L, as it elutes from MonoQ in a similar position as the Pol III-L expressed in *E. coli* (compare Figure 7A with Figure 2).

Example 13 - Demonstration That Peak 1 (Pol III-2) Functions as a Pol III-Type Replicase With *E. coli* Beta Assembled on DNA by *E. coli* Clamp Loader Complex.

To test which peak contained a Pol III-type of polymerase, an assay was used in which the *E. coli* clamp loader complex and beta support formation of full length RFII product starting from *E. coli* SSB coated circular M13mp18 ssDNA primed with a single oligonucleotide. In Figure 8, both Peaks 1 and 2 are stimulated by the *E. coli* clamp loader complex and beta subunit and, in fact, Peaks 2 and 3 are inhibited by these proteins (the quantitation is shown below the gel in the figure). Further, the product analysis in the agarose gel shows full length RFII duplex DNA circles only for peaks 1 and 4. These results, combined with the NEM, pCMB, and

KCl characteristics in Tables 2 and 3 below, suggest that there are two Pol III-type DNA polymerases in *S. aureus* and that these are partially purified in peaks 1 and 4.

Next, it was determined which of these peaks of DNA polymerase activity correspond to DNA polymerases I, II, and III, and which peak is the unidentified DNA polymerase. In the Gram postive bacterium *B. subtilis*, Pol III is inhibited by pCMB, NEM, and 0.15 M NaCl, Pol II is inhibited by KCl, but not NEM or 0.15 M KCL, and Pol I is not inhibited by any of these treatments (Gass et al., "Further Genetic and Enzymological Characterization of the Three *Bacillus subtilis* Deoxyribonucleic Acid Polymerases," J. Biol. Chem., 248:7688-7700 (1973), which is hereby incorporated by reference). Hence, assays were performed in the presence or absence of pCMB, NEM, and 0.15 M KCl (see Tables 2 and 3 below). Peak 3 clearly corresponded to Pol I, because it was not inhibited by NEM, pCMB, or 0.15 M NaCl. Peak 2 correspond to Pol II, because it was not inhibited by NEM, but was inhibited by pCMB and 0.15 M NaCl. Peaks 1 and 4 both had characteristics that mimic Pol III; however, peak 4 elutes on MonoQ at a similar position as Pol III-L expressed in *E. coli* (see Figure 2B). Hence, peak 4 is likely Pol III-L, and peak 1 is likely the unknown polymerase.

Table 2: Expected Characteristics of Polymerases

Polymerase	pCMB	NEM	0.15M KCl
Pol I	not inhibited*	not inhibited	not inhibited
Pol II	inhibited**	not inhibited	not inhibited
Pol III-L	inhibited	inhibited	not inhibited

* Not inhibited is defined as greater than 75% remaining activity
** Inhibited is defined as less than 40% remaining activity

Table 3: Observed Characteristics

Peak	pCMB	NEM	0.15M KCL assignment
Peak1	inhibited	inhibited	new polymerase
Peak2	inhibited	not inhibited	Pol II
Peak3	not inhibited	not inhibited	Pol I
Peak4	inhibited	inhibited	Pol III-L

KCl characteristics in Tables 2 and 3 below, suggest that there are two Pol III-type DNA polymerases in *S. aureus* and that these are partially purified in peaks 1 and 4.

Next, it was determined which of these peaks of DNA polymerase activity correspond to DNA polymerases I, II, and III, and which peak is the unidentified DNA polymerase. In the Gram postive bacterium *B. subtilis*, Pol III is inhibited by pCMB, NEM, and 0.15 M NaCl, Pol II is inhibited by KCl, but not NEM or 0.15 M KCL, and Pol I is not inhibited by any of these treatments (Gass et al., "Further Genetic and Enzymological Characterization of the Three *Bacillus subtilis* Deoxyribonucleic Acid Polymerases," J. Biol. Chem., 248:7688-7700 (1973), which is hereby incorporated by reference). Hence, assays were performed in the presence or absence of pCMB, NEM, and 0.15 M KCl (see Tables 2 and 3 below). Peak 3 clearly corresponded to Pol I, because it was not inhibited by NEM, pCMB, or 0.15 M NaCl. Peak 2 correspond to Pol II, because it was not inhibited by NEM, but was inhibited by pCMB and 0.15 M NaCl. Peaks 1 and 4 both had characteristics that mimic Pol III; however, peak 4 elutes on MonoQ at a similar position as Pol III-L expressed in *E. coli* (see Figure 2B). Hence, peak 4 is likely Pol III-L, and peak 1 is likely the unknown polymerase.

Table 2: Expected Characteristics of Polymerases

Polymerase	pCMB	NEM	0.15M KCl
Pol I	not inhibited*	not inhibited	not inhibited
Pol II	inhibited**	not inhibited	not inhibited
Pol III-L	inhibited	inhibited	not inhibited

* Not inhibited is defined as greater than 75% remaining activity
** Inhibited is defined as less than 40% remaining activity

Table 3: Observed Characteristics

Peak	pCMB	NEM	0.15M KCL assignment
Peak1	inhibited	inhibited	new polymerase
Peak2	inhibited	not inhibited	Pol II
Peak3	not inhibited	not inhibited	Pol I
Peak4	inhibited	inhibited	Pol III-L

Example 14 - Identification and Cloning of *S. aureus dnaE*

This invention describes the finding of two DNA polymerases that function with a sliding clamp assembled onto DNA by a clamp loader. One of these DNA polymerases is likely Pol III-L, but the other has not been identified previously. Presumably, the chromatographic resins used in earlier studies did not have the resolving power to separate the enzyme from other polymerases. This would be compounded by the low activity of Pol III-2. To identify a gene encoding the second Pol III, the amino acid sequences of the Pol III alpha subunit of *Escherichia coli*, *Salmonella typhimurium*, *Vibrio cholerae*, *Haemophilis influenzae*, and *Helicobacter pylori* were aligned using Clustal W (1.5). Two regions about 400 residues apart were conserved and primers were designed for the following amino acid sequences:

Upstream, corresponding in *E. coli* to residues 385-399 (SEQ. ID. No. 43)

```
Leu Leu Phe Glu Arg Phe Leu Asn Pro Glu Arg Val Ser Met Pro
 1               5                  10                  15
```

Downstream, corresponding in *E. coli* to residues 750-764 (SEQ. ID. No. 44)

```
Lys Phe Ala Gly Tyr Gly Phe Asn Lys Ser His Ser Ala Ala Tyr
 1               5                  10                  15
```

The following primers were designed to these two peptide regions using codon preferences for *S. aureus*:

Upstream (SEQ. ID. No. 45)

```
cttctttttg aaagatttct aaataaagaa cgttattcaa tgcc                    44
```

Downstream (SEQ. ID. No. 46)

```
ataagctgca gcatgacttt tattaaaacc ataacctgca aattt                   45
```

Amplification was performed using 2.5 units of *Taq* DNA Polymerase (Gibco, BRL), 100 ng *S. aureus* genomic DNA, 1 mM of each of the four dNTPs, 1 μM of each primer, and 3 mM $MgCl_2$ in 100 μl of *Taq* buffer. Thirty-five cycles of the following scheme were repeated: 94°C, 1 min; 55°C, 1 min; 72°C, 90 sec. The PCR product (approximately 1.1 kb) was electrophoresed in a 0.8 % agarose gel and purified using

a Geneclean III kit (Bio 101). The product was then divided equally into ten separate aliquots and used as a template for PCR reactions, according to the above protocol, to reamplify the fragment for sequencing. The final PCR product was purified using a Quiagen Quiaquick PCR Purification kit, quantitated via optical density at 260 nM, and sequenced by the Protein/DNA Technology Center at Rockefeller University. The same primers used for PCR were used to prime the sequencing reactions.

Next, the following additional PCR primers were designed to obtain more sequence information 3' to the first amplified section.

Upstream (SEQ. ID. No. 47)

```
agttaaaaat gccatatttt gacgtgtttt agttctaat                    39
```

Downstream (SEQ. ID. No. 48)

```
cttgcaaaag cggttgctaa agatgttgga cgaattatgg gg                42
```

These primers were used in a PCR reaction using 2.5 units of *Taq* DNA Polymerase (Gibco, BRL) with 100 ng *S. aureus* genomic DNA as a template, 1mM dNTP's, 1 μM of each primer, and 3 mM $MgCl_2$ in 100 l of *Taq* buffer. Thirty-five cycles of the following scheme were repeated: 94°C, 1 min; 55°C, 1 min; 72°C, 2 min 30 seconds. The 1.6 Kb product was then divided into 5 aliquots, and used as a template in a set of 5 PCR reactions, as described above, to amplify the product for sequencing. The products of these reactions were purified using a Qiagen Qiaquick PCR Purification kit, quantitated via optical density at 260 nm, and sequenced by the Protein/DNA Technology Center at Rockefeller University. The sequence of this product yielded about 740 bp of new sequence 3' of the first sequence.

As this gene shows better homology to the Gram negative Pol III α subunit compared to Gram positive Pol III-L, it will be designated the *dnaE* gene.

Example 15 - Identification and Cloning of *S. aureus dnaX*

The fact that the *S. aureus* beta stimulates Pol III-L and has a ring shape suggests that the Gram postive replication machinery is of the three component type. This implies the presence of a clamp loader complex. This is not a simple

determination to make as the *B. subtilis* genome shows homologs to only two of the five subunits of the *E. coli* clamp loader (*dnaX* encoding gamma, and *holB* encoding delta prime). On the basis of the experiments in this application, which suggests that there is a clamp loader, it was believed that these two subunit homologues are part of the clamp loader for the *S. aureus* beta.

As a start in obtaining the clamp loading apparatus, a strategy was devised to obtain the gene encoding the tau subunit of *S. aureus*. In *E. coli*, the tau and gamma subunits are derived from the same gene. Tau is the full length product, and gamma is about 2/3 the length of tau. Gamma is derived from the *dnaX* gene by what was originally believed to be an efficient translational frameshift mechanism that, after it occurs, incorporates only one unique C-terminal residue before encountering a stop codon. To identify the *dnaX* gene of *S. aureus* by PCR analysis, the *dnaX* genes of *B. subtilis*, *E. coli*, and *H. influenzae* were aligned. Upon comparison of the amino acid sequence encoded by these *dnaX* genes, two areas of high homology were used to predict the amino acid sequence of the *S. aureus dnaX* gene product. PCR primers were designed to these sequences, and a PCR product of the expected size was indeed produced. DNA primers were designed to two regions of high similarity for use in PCR that were about 100 residues apart. The amino acid sequences of these regions were:

Upstream, corresponding to residues 39-48 of *E. coli* (SEQ. ID. No. 49)

```
His Ala Tyr Leu Phe Ser Gly Pro Arg Gly
 1               5                  10
```

Downstream, corresponding to residues 138-148 of *E. coli* (SEQ. ID. No. 50)

```
His Ala Tyr Leu Phe Ser Gly Pro Arg Gly
 1               5                  10
```

The DNA sequence of the PCR primers was based upon the codon usage of *S. aureus*. The primers are as follows:

Upstream (SEQ. ID. No. 51)

```
cgcggatccc atgcatattt attttcaggt ccaagagg                    38
```

Downstream (SEQ. ID. No. 52)

ccggaattct ggtggttctt ctaatgtttt taataatgc 39

The first 9 nucleotides of the upstream primer (SEQ. ID. No. 51) contain a BamHI site, which is underlined, and do not correspond to amino acid codons; the 3' 29 nucleotides correspond to the amino acid sequence of SEQ. ID. No. 49. The EcoRI site of the downstream primer (SEQ. ID. No. 52) is underlined and the 3' 33 nucleotides correspond to the amino acid sequence of SEQ. ID. No. 50.

The expected PCR product, based on the alignment, is approximately 268 bp between the primer sequences. Amplification was performed using 500 ng genomic DNA, 0.5 mM dNTPs, 1 μM of each primer, 1 mM $MgSO_4$, 2 units vent DNA polymerase in 100 μl of vent buffer. Forty cycles were performed using the following cycling scheme: 94°C, 1 min; 60°C, 1 min.; 72°C, 30s. The approximately 300 bp product was digested with EcoRI and BamHI and purified in a 0.7 % agarose gel. The pure digested fragment was ligated into pUC18 which had been digested with EcoRI and BamHI and gel purified in a 0.7 % agarose gel. Ligated products were transformed into *E. coli* competent DH5α cells (Stratagene), and colonies were screened for the correct chimera by examining minipreps for proper length and correct digestion products using EcoRI and BamHI. The sequence of the insert was determined and was found to have high homology to the *dnaX* genes of several bacteria. This sequence was used to design circular PCR primers. Two new primers were designed for circular PCR based on this sequence.

A circular PCR product of approximately 1.6 kb was obtained from a HincII digest of chromosomal DNA that was recircularized with ligase. This first circular PCR yielded most of the remaining *dnaX* gene. The two primers were as follows:

Rightward (SEQ. ID. No. 53)

tttgtaaagg cattacgcag gggactaatt cagatgtg 38

Leftward (SEQ. ID. No. 54)

tatgacattc attacaaggt tctccatcag tgc 33

Genomic DNA (3 μg) was digested with HincII, purified with phenol/chloroform extraction, ethanol precipitated and redissolved in 70 μl T.E. buffer. The genomic DNA was recircularized upon adding 4000 units T4 ligase (New England Biolabs) in a final volume of 100 μl T4 ligase buffer (New England Biolabs) at 16°C overnight. The PCR reaction consisted of 90 ng recircularized genomic DNA, 0.5 mM each dNTP, 100 pmol of each primer, 1.4 mM magnesium sulfate, and 1 unit of elongase (GIBCO) in a final volume of 100 μl elongase buffer (GIBCO). 40 cycles were performed using the following scheme: 94°C, 1 min.; 55°C, 1 min.; and 68°C, 2 min. The resulting PCR product was approximately 1.6 kb. The PCR product was purified from a 0.7 % agarose gel and sequenced directly. A stretch of approximately 750 nucleotides was obtained using the rightward primer used in the circular PCR reaction. To obtain the rest of the sequence, other sequencing primers were designed in succession based on the information of each new sequencing run.

This sequence, when spliced together with the previous 300 bp PCR sequence, contained the complete N-terminus of the gene product (stop codons are present upstream) and possibly lacked only about 50 residues of the C-terminus. The amino terminal region of *E. coli* tau shares what appears to be the most conserved region of the gene as this area shares homology with RFC subunit of the human clamp loader and with the gene 44 protein of the phage T4 clamp loader. An alignment of the N-terminal region of the *S. aureus* tau protein with that of *B. subtilis* and *E. coli* is shown in Figure 10. Among the highly conserved residues are the ATP binding site consensus sequence and the four cystine residues that form a Zn^{2+} finger.

After obtaining 1 kb of sequence in the 5' region of *dnaX*, it was sought to determine the remaining 3' end of the gene. Circular PCR products of approximately 800bps, 600bps, and 1600bps were obtained from Apo I, or Nsi I or Ssp I digest of chromosomal DNA that were recircularized with ligase.

Rightward (SEQ. ID. No. 55)

```
gagcactgat gaacttagaa ttagatatg                                    29
```

Leftward (SEQ. ID. No. 56)

```
gatactcagt atctttctca gatgttttat tc                                32
```

Genomic DNA (3 g) was digested with, Apo I, or Nsi I or Ssp I, purified with phenol/chloroform extraction, ethanol precipitated, and redissolved in 70 l T.E. buffer. The genomic DNA was recircularized upon adding 4000 units of T4 ligase (New England Biolabs) in a final volume of 100 l T4 ligase buffer (New England Biolabs) at 16°C overnight. The PCR reaction consisted of 90 ng recircularized genomic DNA, 0.5 mM each dNTP, 100 pmol of each primer, 1.4 mM magnesium sulfate, and 1 unit of elongase (GIBCO) in a final volume of 100 l elongase buffer (GIBCO). 40 cycles were performed using the following scheme: 94°C, 1 min.; 55°C, 1 min.; 68°C, 2 min. The PCR products were directly cloned into pCR II TOPO vector using the TOPO TA cloning kit (Invitrogen Corporation) for obtaining the rest of the C terminal sequence of *S. aureus dnaX*. DNA sequencing was performed by the Rockefeller University sequencing facility.

Example 16 - Identification and Cloning of *S. aureus dnaB*

In *E. coli*, the DnaB helicase assembles with the DNA polymerase III holoenzyme to form a replisome assembly. The DnaB helicase also interacts directly with the primase to complete the machinery needed to duplicate a double helix. As a first step in studying how the *S. aureus* helicase acts with the replicase and primase, *S. aureus* was examined for presence of a *dnaB* gene.

The amino acid sequences of the DnaB helicase of *Escherichia coli*, *Salmonella typhimurium*, *Haemophilis influenzae*, and *Helicobacter pylori* were aligned using Clustal W (1.5). Two regions about 200 residues apart showed good homology. These peptide sequences were:

Upstream, corresponding to residues 225-238 of *E. coli* DnaB (SEQ. ID. No. 57)

```
Asp Leu Ile Ile Val Ala Ala Arg Pro Ser Met Gly Lys Thr
 1               5                  10
```

Downstream, corresponding to residues 435-449 of *E. coli* DnaB (SEQ. ID. No. 58)

```
Glu Ile Ile Ile Gly Lys Gln Arg Asn Gly Pro Ile Gly Thr Val
 1               5                  10                  15
```

The following primers were designed from regions which contained conserved sequences using codon preferences for *S. aureus*:

Upstream (SEQ. ID. No. 59)

gaccttataa ttgtagctgc acgtccttct atgggaaaaa c 41

Downstream (SEQ. ID. No. 60)

aacattatta agtcagcatc ttgttctatt gatccagatt caacgaag 48

A PCR reaction was carried out using 2.5 units of *Taq* DNA Polymerase (Gibco, BRL) with 100 ng. *S. aureus* genomic DNA as template, 1 mM dNTP's, 1μM of each primer, 3 mM $MgCl_2$ in 100 μl of *Taq* buffer. Thirty-five cycles of the following scheme were repeated: 94°C, 1 min.; 55°C, 1 min.; and 72°C, 1 min. Two PCR products were produced, one was about 1.1 kb, and another was 0.6 kb. The smaller one was the size expected. The 0.6 kb product was gel purified and used as a template for a second round of PCR as follows. The 0.6 kb PCR product was purified from a 0.8% agarose gel using a Geneclean III kit (Bio 101) and then divided equally into five separate aliquots, as a template for PCR reactions. The final PCR product was purified using a Quiagen Quiaquick PCR Purification kit, quantitated via optical density at 260 nM, and sequenced by the Protein/DNA Technology Center at Rockefeller University. The same primers used for PCR were used to prime the sequencing reaction. The amino acid sequence was determined by translation of the DNA sequence in all three reading frames, and selecting the longest open reading frame. The PCR product contained an open reading frame over its entire length. The predicted amino acid sequence shares homology to the amino acid sequences encoded by *dnaB* gene of other organisms.

Additional sequence information was determined using the circular PCR technique. Briefly, *S. aureus* genomic DNA was digested with various endonucleases, then religated with T4 DNA ligase to form circular templates. To perform PCR, two primers were designed from the initial sequence.

First primer (SEQ. ID. No. 61)

gatttgtagt tctggtaatg ttgactcaaa ccgcttaaga accgg 45

Second primer (SEQ. ID. No. 62)

atacgtgtgg ttaactgatc agcaacccat ctctagtgag aaaatacc 48

The first primer matches the sequence of the coding strand and the second primer matches the sequence of the complementary strand. These two primers are directed outwards from a central point, and allow determination of new sequence information up to the ligated endonuclease site. A PCR product of approximately 900 bases in length was produced using the above primers and template derived from the ligation of *S. aureus* genomic DNA which had been cut with the restriction endonuclease Apo I. This PCR product was electrophoresed in a 0.8% agarose gel, eluted with a Qiagen gel elution kit, divided into five separate aliquots, and used as a template for reamplification by PCR using the same primers as described above. The final product was electrophoresed in an 0.8% agarose gel, visualized via staining with ethidium bromide under ultraviolet light, and excised from the gel. The excised gel slice was frozen, and centrifuged at 12,000 rpm for 15 minutes. The supernatant was extracted with phenol/chloroform to remove ethidium bromide, and was then cleaned using a Qiagen PCR purification kit. The material was then quantitated from its optical density at 260 nm and sequenced by the Protein/DNA Technology Center at the Rockefeller University.

The nucleotide sequence contained an open reading frame over its length, up to a sequence which corresponded to the consensus sequence of a cleavage site of the enzyme Apo I. Following this point, a second open reading frame encoded a different reading frame up to the end of the product. The inital sequence information was found to match the inital sequence and to extend it yet further towards the C-terminus of the protein. The second reading frame was found to end in a sequence which matched the 5'-terminus of the previously determined sequence and, thus, represents an extension of the sequence towards the N-terminus of the protein.

Additional sequence information was obtained using the above primers and a template generated using *S. aureus* genomic DNA circularized via ligation with T4 ligase following digestion with Cla I. The PCR product was generated using 35 cycles of the following program: denaturation at 94°C for 1 min.; annealing at 55°C for 1 min.; and extension at 68°C for 3 minutes and 30 s. The PCR products were electrophoresed in a 0.8% agarose gel, eluted with a Qiagen gel elution kit, divided into five separate aliquots, and used as a template reamplification via PCR with the same primers described above. The final product was electrophoresed in an 0.8%

agarose gel, visualized via staining with ethidium bromide under ultraviolet light, and excised from the gel. The excised gel slice was frozen, and centrifuged at 12,000 rpm for 15 min. The supernatant was cleaned using a Qiagen PCR purification kit. The material was then quantitated via optical density at 260 nm and sequenced by the Protein/DNA Technology Center at Rockefeller University. The open reading frames continued past 500 bases. Therefore, the following additional sequencing primers were designed from the sequence to obtain further information:

First primer (SEQ. ID. No. 63)

```
cgttttaatg catgcttaga aacgatatca g                    31
```

Second primer (SEQ. ID. No. 64)

```
cattgctaag caacgttacg gtccaacagg c                    31
```

The N-terminal and C-terminal nucleotide sequence extensions generated using this circular PCR product completed the 5' region of the gene (encoding the N-terminus of DnaB); however, a stop codon was not reached in the 3' region and, thus, a small amount of sequence is still needed to complete this gene.

The alignment of the *S. aureus dnaB* with *E. coli dnaB* and the *dnaB* genes of *B. subtilis* and *S. typhimurium* is shown in Figure 11.

Example 17 - Identification and Cloning of *S. aureus holB*

The *S. aureus holB* was identified by searching the *S. aureus* database with the sequences of *S. pyogenes* δ' subunit. The *S. aureus holB* encodes a 253 residue protein of about 28 kDa. The *holB* gene was amplified by PCR using an upstream 69-mer primer as follows:

Upstream Primer (SEQ. ID. No. 65):

```
ggataacaat tccccgctag caataatttt gtttaacttt aagaaggaga tatacccatg    60
gatgaacag                                                            69
```

which contains an *NcoI* site (underlined), and a downstream 39-mer primer as follows:

Downstream Primer (SEQ. ID. No. 66):
aattttaaag gatccgtgta taatattcta attttcccg 39

which contains a *BamHI* site (underlined). The PCR product was digested with *NcoI* and *BamHI*, purified, and ligated into the *NcoI* and *BamHI* sites of pET11a to produce plasmid pETSaholB.

Example 18 - Purification of *S. aureus* δ'

The pETSaholB plasmid of Example 17 was transformed into *E. coli* BL21(DE3)*recA*. A single colony was used to innoculate 2L of LB media supplemented with 200 μg/ml ampicillin. Cells (2L) were grown at 37°C to OD_{600}=0.5 at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. After 16 hr of induction, cells were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5), 10% sucrose, 1 M NaCl, 30 mM spermidine, 5 mM DTT, and 2 mM EDTA. Cells were lysed by two passages through a French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min at 4°C. Ammonium sulfate (0.3 g/ml) was added to the clarified lysate. The pellet was backwashed in 30 ml buffer A containing 0.1 M NaCl and 0.24 g/ml ammonium sulfate using a Dounce homogenizer, then the pellet was recovered by centrifugation. The resulting pellet was resuspended in 20 ml of buffer A and dialyzed against buffer A. The dialyzed protein was applied to a 20 ml FFQ Sepharose column equilibrated in buffer A and eluted with a 200 ml linear gradient of 0 - 500 mM NaCl in buffer A; 80 fractions were collected. Peak fractions (54 - 75) were combined (72 mg) and dialyzed against buffer A. The δ' preparation was aliquoted and stored frozen at -80°C.

Example 19 - Identification and Cloning of *S. aureus holA*

The *S. aureus holA* gene was identified by searching the *S. aureus* database with the sequences of *E. coli* and *S. pyogenes* δ subunits. The *S. aureus holA*

gene encodes a 288 residue protein of about 32 kDa. The *holA* gene was amplified by PCR using an upstream 28-mer primer as follows:

Upstream Primer (SEQ. ID. No. 67):

gggagtttgt aatccatgga tgaacagc 28

which contains a *NcoI* site (underlined), and a downstream 37-mer primer as follows:

Downstream Primer (SEQ. ID. No. 68):

ctgaacacct attaccctag gcatctaact cacaccc 37

which contains a *BamHI* site (underlined). The PCR product was digested with *NcoI* and *BamHI*, purified, and ligated into the *NcoI* and *BamHI* sites of pET11a to produce plasmid pETSaholA.

Example 20 - Purification of *S. aureus* δ

The pETSaholA plasmid of Example 19 was transformed into *E. coli* NovaBlue (*recA1* $lac[F'proA^{+}B^{+}\ lac^{q}Z\Delta M15::Tn10(Tc^{R})]$) (Novagen). A single colony was used to innoculate 12L of LB media supplemented with 200 μg/ml ampicillin. Cells (12L) were grown at 37°C to OD_{600}=0.5 at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. After 16 hr of induction, cells were collected by centrifugation, resuspended in 50 mM Tris-HCl (pH 7.5), 10% sucrose, 1M NaCl, 30 mM spermidine, 5 mM DTT, and 2 mM EDTA. Cells were lysed by two passages through a French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min at 4°C. Ammonium sulfate (0.3 g/ml) was added to the clarified lysate. The resulting pellet was resuspended in 250 ml of buffer A. The dialyzed protein was applied to a 100 ml FFQ Sepharose column equilibrated in buffer A and eluted with a 1000 ml linear gradient of 0 - 500 mM NaCl in buffer A; 80 fractions were collected. Peak fractions (40-49) were combined (65 mg) and dialyzed against buffer A. The dialyzed protein was applied to a 8 ml MonoQ Sepharose column equilibrated in buffer A and eluted with a 80 ml linear gradient of 0

- 500 mM NaCl in buffer A; 80 fractions were collected. Peak fractions of the δ preparation were stored frozen at -80°C.

Example 21 - Consitution of a Processive *S. aureus* DNA Polymerase III Enzyme from Three Components

The PolC (alpha-large) requires the β clamp for processivity, which in turn requires the clamp loader (τδδ') for assembly onto DNA. The *S. aureus* clamp loader, τδδ' complex, was assembled by mixing the three proteins as follows: 400 μg of τ and 80 μg each of δ and δ' were mixed in buffer A containing no NaCl and preincubated at 15°C for 10 min. The mixture was injected onto a 1 ml MonoQ column equilibrated in buffer A, and then eluted with a 30 ml linear gradient of 0-500 mM NaCl in buffer A; 60 fractions were collected. Fractions were analyzed in a 10% SDS-polyacrylamide gel stained with Coomassie Blue. Peak fractions (40-50) were combined and concentrated using a Centricon 30 concentrator.

The ability of the three components to work together to form the processive Pol III was tested by determining whether τδδ' and β clamp could confer the ability of PolC to completely extend a single primer full circle around a large 7.2 kb circular M13mp18 ssDNA genome. Replication reaction contained 70 ng (25 fmol) on singly primed M13mp18 ssDNA, 20 ng *S. aureus* β, 50 ng *S. aureus* PolC, either 30 ng or 90 ng of *S. aureus* τδδ' (when indicated), and 0.82 μg of *S. pyogenes* SSB in 24 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 8 mM $MgCl_2$, 40 μg/ml BSA, and 60 mM each of dGTP and dCTP. Reactions were pre-incubated for 2 min at 37°C to assemble protein complexes on the primer terminus. DNA synthesis was initiated upon addition of 1.5 μl dATP and ^{32}P-TTP (specific activity 2,000-4,000 cpm/pmol) and synthesis was allowed to proceed for 1 min before being quenched with an equal volume (25 μl) of a solution of 1% SDS and 40 mM EDTA. One-half of the quenched reaction was analyzed for total DNA synthesis using DE81 paper as described, and the other half was analyzed by agarose gel phoresis. An autoradiogram of the agarose gel analysis of the replication products is depicted in Figure 13, which shows that the presence of PolC and β, but absence of τδδ' (lane 1) gives no full length circular duplex (RFII). However, in the

presence of τδδ' (lanes 2 and 3), full length circular duplex DNA (RFII) is produced, as expected for the action of a processive Pol III holozyme.

Example 22 - General Induction/Purification Conditions for *S. pyogenes*

The purification protocols for *S. pyogenes* proteins were performed using following standardized conditions. Cells were grown from a single colony, freshly transformed overnight. Cells were grown in 200 μg/ml Ampicillin to OD600=0.3-0.4, at which point cultures were chilled prior to addition of IPTG (to a final concentration of 0.5 mM) and were allowed to incubate for 16 hrs at 15°C. Following this, all procedures were performed at 4°C. Cell paste (1-2 g/liter of culture) was resuspended (10 ml/g cell paste) in 50 mM Tris-HCl (pH 7.5)/10% Sucrose/1 M NaCl/5 mM DTT/ 30 mM Spermidine/1X Heat lysis buffer (50 mM Tris-HCl (pH 7.5), 1% Sucrose, 100 mM NaCl, 2 mM EDTA). Cells were lysed by two passages through the French Press (15,000 psi) followed by centrifugation at 14,000 rpm at 4 °C. Ammonium sulfate, when added to the cleared lysate, was added gradually. Precipitate was allowed to settle on ice for a minimum of 30 min prior to collection by centrifugation. Protein pellets were resuspended in buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM DTT, 10% glycerol) and dialyzed for over 3 hours in the same buffer. Column design is based on the manufacturer's suggested capacities: Fast Flow Q (FFQ) and MonoQ are 20 mg protein /ml resin, Heparin-Affigel agarose is 1.2 mg protein/ml resin. Elution was performed using 10 column volume (c.v.) gradients, and the entire gradient elution profile was collected in 80 fractions. Unless mentioned otherwise all columns were equilibrated and eluted with buffer A.

Example 23 - Identification of a *S. pyogenes holA* gene Encoding a Functional Delta Subunit and Purification of the Delta Subunit

Alignment of *E. coli* delta subunit with 10 other putative *holA* products from unfinished genome databases of Gram negative bacteria indicates a region of conserved amino acid sequence. Amino acids Q140 to L230 of *E. coli* delta were used to search the *B. subtilis* genome database for a Gram positive delta homolog. This search revealed *yqeN*, a potential reading frame of unknown function, as the

highest scoring sequence. Although the score was low, it was treated as a candidate for Gram positive delta. The alignment with *E. coli* delta is shown in Figure 12A. A *Streptococcus pyogenes* genome database was searched with *yqeN*. Two contigs which represent N- (contig 206) and C- (contig 264) termini of *S. pyogenes* delta subunit were identified. The alignment of the putative *S. pyogenes holA* with *B. subtilis yqeN* is shown in Figure 12B. The following primers were used to obtain PCR products for delta subunit:

holA Upstream (SEQ. ID No. 69)

ggagcagatt gcttttgata catatgattg gcctattc 38

holA Downstream (SEQ. ID No. 70)

ttgtctccgc atcaaactgg gatccaagag catcatacgc gtatgg 46

These primers were used to amplify the *holA* gene from *S. pyogenes* genomic DNA. The PCR product was digested with NdeI and BamHI, purified and ligated into the pET11a vector to produce pET11a.S.p. holA.

The pET11a.S.p.holA plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 12L LB broth supplemented with 200 µg/ml Ampicillin. Cells were grown at 37°C to OD600=0.5, at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning, cells were collected by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/1M NaCl/30 mM Spermidine/5 mM DTT. Cells were lysed by two passages through the French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min. The supernatant was decanted and ammonium sulfate was added to a final concentration of 0.226 g/ml. The resulting pellet was collected by centrifugation and resuspended in 20 ml of buffer A. The resuspended pellet was dialyzed against buffer A containing no salt. The dialyzed protein (500 mg) was loaded onto a FFQ- Sepharose (35 ml) column and eluted with a linear gradient from 0 - 500 mM NaCl (10 c.v.). The peak fractions (21-45) were combined and dialyzed against buffer A (0 NaCl) for 3 hrs, then diluted to a conductivity of 50 mM NaCl and loaded (160 mg) onto a 120 ml Heparin-Affigel

column. Protein was eluted with a linear gradient of 0-500 mM NaCl (10 c.v.). The fractions containing the least contaminants (39-51) were precipitated with ammonium sulfate (0.226 g), collected by centrifugation, resuspended 5 ml of buffer A, and dialyzed in buffer A containing 200 mM NaCl. The delta subunit was stored at -80°C. The final delta subunit preparation is shown in the lane marked δ of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 65 mg.

Example 24 - Identification of *S. pyogenes holB* Encoding Delta Prime and Purification of the Delta Prime Subunit

A search of the *S. pyogenes* genome database with the predicted *B. subtilis* delta prime amino acid sequence revealed a DNA sequence in contig #209 (previously known as contig # 210) that predicted a high scoring match for a gene encoding a delta prime protein. The following primers were used to obtain PCR products for *holB*:

holB Upstream (SEQ. ID. No. 71)
gcctaggata agggagggta catatggatt tagcgc 36

holB Downstream (SEQ. ID. No. 72)
cgggcaagtc ttttgacaag cttcggatcc ccataacgaa ttcc 44

The PCR product obtained from these primers was digested with NdeI and BamHI, purified and ligated into the pET11a vector to produce pET11a.S.p. holB.

The pET11a.S.p.holB plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 12L LB broth supplemented with 200 µg/ml Ampicillin. Cells were grown at 37°C to O.D.600=0.4, at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning, cells were collected by centrifugation and resuspended in 100 ml 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer. Lysis was initiated upon addition of 0.4 mg/ml lysozyme followed by a 1 hr incubation on ice. Lysate was clarified by centrifugation at 13,000 rpm for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 0.3 g/ml. The protein pellet was resuspended in

buffer A(0.1 M NaCl) + 0.24 g/ml ammonium sulfate and clarified by centrifugation. The resulting protein pellet was resuspended in 20 ml of buffer A and dialyzed against buffer A. The dialyzed protein (450 mg) was loaded onto a 30 ml FFQ- Sepharose column and eluted with a linear gradient from 0 - 500 mM NaCl. The peak fractions were combined (fr# 20-30 containing 130 mg) and dialyzed against buffer A and loaded (70 mg) onto a 50 ml Heparin-Affigel column. Protein was eluted with a linear gradient of 0-500 mM NaCl. Delta prime binds weakly to both resins and elutes in the beginning of the gradient. This delta prime subunit was stored frozen at - 80°C. The final delta prime subunit preparation is shown in lane marked δ' of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 40 mg.

Example 25 - Identification of the *S. pyogenes dnaX* Gene and Purification of the Tau Subunit

A search of the *S. pyogenes* genome database with the putative *B. subtilis* tau amino acid sequence revealed a DNA sequence in contig #284 (previously known as contig # 289) with a high scoring match which predicted a gene encoding for a tau subunit protein. A set of PCR primers to 5'- and 3'- termini of the putative gene sequence were designed to include restriction enzyme recognition sequences for NdeI and BamHI sites, respectively. These primers are:

dnaX Upstream (SEQ. ID. No. 73)

```
ggagttaaaa acatatgtat caagctcttt atc                    33
```

dnaX Downstream (SEQ. ID. No. 74)

```
cgtgggtaag ggcaaaacgg atcccttatg tatttcag                38
```

A PCR product obtained with the above primers was digested with NdeI and BamHI, purified and ligated into pET11a vector to produce pET11a.S.p.dnaX.

The pET11a.S.p.dnaX plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 24L LB broth supplemented with 200 µg/ml Ampicillin. Cells were grown at 37°C to O.D.600=0.5, at which point the temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning,

cells were collected by centrifugation and resuspended in 200 mls of 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/1M NaCl/30 mM Spermidine/5 mM DTT/5 mM EDTA. Cells were lysed by two passages through the French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min. The supernatant (2.4 gm) was dialyzed against buffer A containing 50 mM NaCl, loaded onto a 120 ml FFQ column (without ammonium sulfate precipitation) and eluted with a linear gradient of 100-700 mM NaCl. The peak fractions (fr# 41-55) were combined, diluted with buffer A containing no salt (a dilution of 1/5) to a conductivity of 100 mM NaCl, loaded (310 mg) onto a 300 ml Heparin-Affigel column, and eluted with a linear gradient of 100-500 mM NaCl. The peak fractions (fr# 21-36) were combined, dialyzed against buffer A, loaded (87 mg) onto 10 ml FFQ column, and eluted as described for the first FFQ column. The peak fractions (fr# 27-41) were concentrated by centrifugation in Centriprep 30 filtration unit and frozen at -80°C. The final tau subunit preparation is shown in the lane marked τ of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 103 mg.

Example 26 - Identification of the *S. pyogenes dnaN* Gene and Purification of the Beta Subunit

A search of the *S. pyogenes* genome database with the putative *B. subtilis* beta subunit amino acid sequence revealed a DNA sequence (contig # 266) with a high scoring match which predicted a gene encoding for a beta subunit protein. A set of PCR primers to 5'- and 3'- termini of the putative gene sequence were designed to include restriction enzyme recognition sequences for NdeI and BamHI, respectively. The primers were:

dnaN Upstream (SEQ. ID. No. 75)

```
ggagttcata tgattcaatt ttcaaattaa tcgc                    34
```

dnaN Downstream (SEQ. ID. No. 76)

```
tatcagctcc tggatccagt accttccatt gattagcc                38
```

A PCR product obtained with these primers was digested with NdeI and BamHI, purified and ligated into pET16b vector to produce pET16b.S.p.dnaN.

The pET16b.S.p.dnaN plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 15L LB broth supplemented with 200 μg/ml Ampicillin. Cells were grown at 37°C to O.D.600=0.4, at which the point temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning, cells were collected by centrifugation and resuspended in 100 ml 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/1 M NaCl/5 mM DTT/ 30 mM Spermidine/5 mM EDTA. Cells were lysed by two passages through the French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 0.3 g/ml. The resulting protein pellet was resuspended and dialyzed against buffer A containing 50 mM NaCl. The dialyzed protein (300 mg) was loaded onto a 45 ml FFQ- Sepharose column and eluted with a linear gradient from 50 - 500 mM NaCl. The peak fractions (16-30) were combined, dialyzed against buffer A containing 50 mM NaCl, loaded onto a 25 ml EAH-Sepharose column, and eluted with a linear gradient of 50-500 mM NaCl. The fractions containing the least contaminants were combined into two pools (pool I 10-17, pool II 19-27). Each pool was further purified on a 8 ml MonoQ column (performed under conditions described for the FFQ column above). The final beta subunit preparation is shown in the lane marked β of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 48 mg.

Example 27 - Identification of the *S. pyogenes polC* Gene and Purification of the Alpha-Large Polymerase Subunit

A search of the *B. subtilis* genome database with the *E. coli* alpha subunit amino acid sequence revealed two DNA sequences with a high scoring match which predicted two genes encoding alpha-like polymerase subunits. The DNA sequence with the second highest scoring match which encoded the largest of the two polymerase subunits also appeared to encode for the epsilon exonuclease domain at the N- terminus of the putative alpha subunit. A search of the *B. subtilis* genome database with *S. pyogenes* DNA sequence confirmed this nucleotide sequence to encode the Gram positive homolog of the *E. coli* replicative polymerase subunit (alpha). This Gram negative alpha-like subunit lacked homology to epsilon. The gene encoding the large alpha polypeptide sequence (alpha-large) will be referred to as

the product of the *polC* gene and the gene encoding the smaller Gram-negative alpha-like polymerase (alpha-small) will be referred to as the product of the *polE* or *dnaE* gene (see Example 28).

The alpha-large polymerase polypeptide is a product of two overlapping contigs; contig #197 (renamed #193) encodes the N-terminal 630 amino acids, and contig #278 (renamed #273) encodes the C-terminal 1392 amino acids. The putative Open Reading Frame generates a 1464 amino acid polypeptide (SEQ. ID. No. 18). Since the *polC* nucleotide sequence contained several NdeI sites, a primer was designed to mutate two restriction endonuclease sites in the pET11a nucleotide sequence upstream of the N-terminus of the gene; an XbaI restriction site was mutated to an NheI restriction site and an NdeI restriction site at the starting ATG was removed. A 74mer primer which spans from mutated XbaI site upstream of T7 promoter includes NheI site, rbs site (ribosome binding site), mutated NdeI site and first 10 amino acid codons of *polC* gene sequence. The following primers were used in a PCR reaction to amplify *polC* gene from *S. pyogenes* genomic DNA:

polC Upstream (SEQ. ID. No. 77)

```
ggataacaat tccccgctag caataatttt gtttaacttt aagaaggaga tatacccatg  60
tcagatttat tcgc                                                    74
```

polC Downstream (SEQ. ID. No. 78)

```
cggtgtctct atctaaatga ctcatttggg atcctcgctt tatacggtat gtcacag     57
```

Elongase (BRL) produced the best amplification results. PCR reaction conditions were: 5 μg genomic DNA, 20 ng of each primer, 1 ml Elongase, 60 μM each dNTP, in 100 ml Elongase reaction buffer for 1 min at 94°C, 1 min at 55°C, and 6 min at 60°C repeated for 40 cycles. The resulting 4000 bp PCR fragment was digested with NheI and BamHI, purified and ligated into the pET11a vector (digested with XbaI and BamHI) to produce pET11a.S.p.polC.

The pET11a.S.p.polC plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 24L LB broth supplemented with 200 μg/ml Ampicillin. Cells were grown at 37°C to OD600=0.4 at which point temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning, cells

(12g) were collected by centrifugation and resuspended in 100 ml 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/1 M NaCl/5mM DTT/30 mM Spermidine/5 mM EDTA. Cells were lysed by two passages through the French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 0.226 g/ml. The precipitate was collected by centrifugation. The protein pellet (220 mg resuspended in buffer A) was dialyzed against buffer A containing 150 mM NaCl, loaded onto an 8 ml FFQ column equilibrated with buffer A containing 150 mM NaCl, and eluted with a linear gradient of buffer A containing 150-600mM NaCl. The fractions containing the least contaminants (fr# 42-64) were combined and precipitated with ammonium sulfate (0.226 g/ml). The precipitate was collected by centrifugation and resuspended in buffer A (10 mg/ml in 5 ml). A fraction (1 ml=10mgs) of the concentrated protein was dialyzed, loaded onto 10 ml ssDNA-agarose column, and eluted with a linear gradient of 50-500 mM NaCl. The peak fractions (fr# 30-50) were combined and concentrated with ammonium sulfate (as above). The final alpha-large subunit preparation is shown in lane marked α_L of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield= 4 mgs.

Example 28 - Identification of the *S. pyogenes dnaE* Gene and Purification of the Alpha-Small Polymerase

A search of the *B. subtilis* genome database using the *E. coli* alpha subunit amino acid sequence revealed two DNA sequences with a high scoring match which predicted two genes encoding for alpha-like polymerase subunits. The DNA sequence with the highest scoring match encodes a smaller alpha polymerase which does not contain an exonuclease domain. The putative short alpha DNA sequence is a product of the open reading frame in contig #253 of the *S. pyogenes* genome database. A set of PCR primers to 5'- and 3'-termini of the putative gene sequence were designed to include restriction enzyme recognition sequences for NdeI and BamHI, respectively. The primers were:

α -short Upstream (SEQ. ID. No. 79)

gggaacaaga taaccaagga ggaacccatg gttgctcaac ttg 43

α -short Downstream (SEQ. ID. No. 80)

cgaatagcag cgttcatacc aggatcctcg ccgccactgg 40

A PCR product obtained with these primers was digested with NdeI and BamHI, purified and ligated into pET11a vector to produce pET11a.S.p.dnaE.

The pET11a.S.p.dnaE plasmid was transformed into the BL21(DE3)RecA- strain of E. coli. A single colony from an overnight transformation was used to innoculate 12L LB broth supplimented with 200 μg/ml Ampicillin. Cells were grown at 37°C to OD600=0.4, at which point temperature was lowered to 15°C and 0.5 mM IPTG was added. Induction proceeded for 16 hrs. In the morning, cells were collected by centrifugation and resuspended in 100 mls 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/5 mM DTT/30 mM Spermidine/1M NaCl/5 mM EDTA. Cells were lysed by two passages through the French press (15,000 psi), followed by centrifugation at 13,000 rpm for 30 min. Ammonium sulfate was added to the supernatant to a final concentration of 0.226 g/ml. The precipitate was collected by centrifugation. The protein pellet (resuspended in buffer A) was then dialyzed against buffer A. The dialyzed protein (600 mg) was loaded onto a 30 ml FFQ and eluted with a linear gradient of buffer A containing 50-500 mM NaCl. The peak fractions (200 mg in fr # 70-79) were dialyzed and loaded onto a 100 ml Heparin-Affigel column. The fractions containing the least contaminants (100 mg from fr # 18-30) were pooled and dialyzed against buffer A containing 300 mM NaCl. The dialysate (50 mg) was loaded onto a 50 ml ssDNA-agarose column and eluted with a linear gradient of 300mM - 1M NaCl. The final alpha-small subunit preparation is shown in lane marked α_S of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 25 mg.

Example 29 - Identification of the *S. pyogenes* ssb Gene and Purification of the Single Strand DNA-Binding Protein

Search of the *S. pyogenes* genome using the *B. subtilis* SSB amino acid sequence identified a polypeptide in contig #230(212) as having highest homology to single strand binding protein of several Gram negative bacteria. This contig lacked the first 26 amino acids at the N-terminus. Circular PCR was employed to identify the DNA encoding the N-terminus of the putative SSB protein. *S. pyogenes* genomic

DNA was digested overnight with ApoI (5 µg chromosomal DNA in a 50 µl reaction). The DNA was extracted with phenol and precipitated with ethanol. The ApoI digested chromosomal DNA was self-ligated to generate circular template for future use in the circular PCR. A circular PCR was performed with primers designed to anneal back-to-back to amplify circularized ApoI reaction fragments. The primers were:

ssb.circ Upstream (SEQ. ID. No. 81)

```
accattttgg cttttaaagg tacggttaac agcaagtgtg aaggtagcc          49
```

ssb.circ Downstream (SEQ. ID. No. 82)

```
gaacgcgagg cagatttcat taactgtgtg atctggcg                      38
```

The PCR reaction conditions were as follows: 100 ng circularized *S. pyogenes* genomic DNA, 20 ng each primer, 1 ml Elongase, 60 µM each dNTP, 100 l Elongase reaction buffer. Amplification was performed for 40 cycles as follows: denature, 1 min at 94°C; anneal, 1 min at 55°C; and extend, 5 min at 68°C. PCR products were cloned into the Topo TA vector following instructions of the manufacturer (Promega). Several positive clones were sequenced to obtain N-terminal nucleotide sequence. This information lead to design of the following primers with which the use of a standard PCR reaction generated whole *ssb* gene products. The primers were:

ssb Upstream (SEQ. ID. No. 83)

```
tttaaaagag ggtagcatat gattaataat gtagtactag ttggtcgc            48
```

ssb Downstream (SEQ. ID. No. 84)

```
tttaaattta aacctaggtt caatccattc tgactagaat ggaagatcgt c        51
```

The resulting PCR product was digested with NdeI and BamHI, purified and ligated into pET11a vector to produce pET11a.S.p. ssb.

The pET11a.S.p.ssb plasmid was transformed into the BL21(DE3)RecA- strain of *E. coli*. A single colony from an overnight transformation was used to innoculate 12L LB broth supplemented with 200 µg/ml Ampicillin. Cells

were grown at 37°C to OD600=0.5, at which point 0.5 mM IPTG was added. At the end of the 3 hr induction, cells were collected by centrifugation and resuspended in 100 ml of 50 mM Tris-HCl (pH 7.5)/ 10% Sucrose /1X Heat Lysis Buffer/5 mM DTT/5 mM EDTA. The cell lysis was initiated upon addition of 0.4 mg/ml lysozyme followed by a 1 hr incubation on ice. The lysate was clarified by centrifugation at 13,000 rpm for 30 min. The SSB protein was significantly purified by sequential fractionation with ammonium sulfate in the following manner. Solid ammonium sulfate was added to the clarified lysate to a final concentration of 0.24 g/ml and the precipitated protein was collected by centrifugation at 13,000 rpm for 30 min. The resulting pellet was homogenized in buffer A(0.1 M NaCl) + 0.24 g/ml ammonium sulfate and the precipitate was collected by centrifugation. This procedure was repeated with buffer A(0.1 M NaCl) + 0.2 g/ml ammonium sulfate, buffer A(0.1 M NaCl + 0.15 g/ml ammonium sulfate, and buffer A(0.1 M NaCl) + 0.13 g/ml ammonium sulfate. The final pellet was resuspended in buffer A + 0.15 M NaCl and dialyzed against the same buffer. The resulting pellet was resuspended in buffer A and dialyzed against buffer A containing 500 mM NaCl. The dialysate (300 mg) was diluted to 0.15 M NaCl before it was loaded onto a 20 ml MonoQ column and eluted with a linear gradient of 0.15 M - 0.5 M NaCl in buffer A. The SSB protein elutes in the very beginning of the gradient. The peak fractions were combined (150 mg in fractions 16-30), diluted to 0.05 M NaCl, loaded onto a 10 ml ssDNA-agarose column, and eluted with 0.5 M NaCl. The peak fractions (32-62) were combined and frozen. The SSB was further purified over a MonoQ column to remove contaminating polymerase activity. The final single strand DNA binding protein preparation is shown in lane marked ssb of the Coomassie Blue stained SDS-polyacrylamide gel of Figure 14. Yield = 120 mg.

Example 30 - First Demonstration that *S. pyogene holA* Encodes a Delta Subunit Involved In Replication: Assembly of τδδ' Complex

Gel filtration is a standard analytical technique to demonstrate direct protein-protein interaction. Purified τ, δ, δ' proteins were used to examine whether they form a protein complex assembly. Gel filtration of τ mixed with either δ, δ',or both δ and δ' was performed using an HR 10/30 Superose 6 column equilibrated with

buffer A containing 100 mM NaCl. Either δ (200 μg), δ' (200 μg), or a mixture of δ and δ' (200 μg each) was incubated for 30 min at 15°C in 100 μl of buffer A containing 100 mM NaCl, and the entire mixture was injected onto the column. The mixture was resolved on the column by collection of 170 μl fractions after the initial void (6.6 μl) volume was collected. Fractions were analyzed by 10% SDS-polyacrylamide gels (30 μl/lane) stained with Coomassie Blue.

The results, in Figure 15, demonstrate that under these conditions the τ protein exhibits no (weak) interaction with the delta (Figure 15B) and the delta prime subunits (Figure 15C) individually, and yet assembles readily into a complex when all the subunits are mixed in the reaction (Figure 15A). The τ protein was mixed with a 2-fold molar excess of each δ and δ', then gel filtered. A complex of τδδ' was formed as demonstrated by coellution of δ and δ' with τ (fr# 22-30) whereas excess δδ' complex elutes in later fractions (fr#38-46). To determine whether individual δ or δ' subunits interact with τ, the τ subunit was mixed with either δ or δ' and then gel filtered. The results demonstrate that a gel filterable complex does not form when τ is mixed with δ (Figure 15B) or δ' (Figure 15C) subunits individually, as indicated by the absence of these subunits in the τ containing fractions (fr#20-26). Therefore, it appears that the presence of both δ and δ' subunits is essential for the formation of the τδδ' complex.

Example 31 - Second Demonstration that *S. pyogenes holA* Encodes Delta: Functional Assembly of β on DNA

Gel filtration was used to demonstrate that the τ, δ, δ' proteins form a functional clamp loading complex which is able to load the β clamp onto a circular DNA molecule. The reaction contained 0.5 pmol of gp2 nicked pBluescript plasmid (a circular double strand plasmid with a single nick produced by M13 gp2 protein), 1 pmol [^{32}P]β, 0.5 pmol τδδ' complex, 0.25 pmol of either δ, δ', τ were used in individual experiments when a subassembly of the complex was tested (τδ, τδ', δδ') in 75 μl buffer B (20 mM Tris-HCl (pH 7.5), 20 % glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 8 mM $MgCl_2$). β was incubated with nicked DNA for 10 min at 37°C either alone, or in combination with various assemblies of the τ complex. All gel

filtration experiments were performed at 4°C. The reaction mixtures were applied to a 5 ml column of Bio-Gel 15M (Bio-Rad) equilibrated in buffer B containing 100 mM NaCl. Fractions of 170 µl were collected and quantitated in the Scintillation counter.

The results, in Figure 16, demonstrate that the assembly of the ring onto a circular DNA molecule requires the presence of τ, δ, and δ' proteins (Figure 16A). In absence of any one of the subunits, loading onto DNA does not occur (Figure 16B-E). The clamp loader complex (τδδ') can be supplied as a mixture of τ, δ, δ' subunits or as an assembled complex (purified from unassembled subunits by gel filtration, or by ion exchange chromatography on MonoQ). Proteins bound to the large DNA molecule elute in the early fractions (void fr# 10-17) and resolve from free proteins that elute in later fractions (fr# 18-35).

Example 32 - The τ Subunit Product of the *dnaX* Gene Binds α -large

The interaction of *S. pyogenes* α and τ proteins was examined by analyzing a mixture of the proteins by gel filtration. Gel filtration of τ, α -large or a mixture of α-large and τ was performed using an HR 10/30 Superose 6 column equilibrated with buffer A containing 100 mM NaCl. Either α-large (400 µg) (200 µM) or a mixture of α-large and τ was incubated for 30 min at 15°C in 100 µl of buffer A containing 100 mM NaCl, and the entire mixture was injected onto the column. The mixture was resolved on the column by collection of 170 µl fractions after the initial void (6.6 ml) volume was collected. Fractions were analyzed by 10% SDS-polyacrylamide gels (30 µl/lane) stained with Coomassie Blue.

The results show a complex of $\alpha_L\tau$ was formed as demonstrated by coellution of α-large and τ (fr# 30-38) proteins (Figure 17A) compared to the elution profile of individual proteins (Figure 17B-C). Also, the migration of the τ in the $\alpha_L\tau$ complex changes significantly to a larger complex (4 fractions, from fr# 37 to fr# 33).

Example 33 - Formation of $\alpha_L\tau\delta\delta'$ Complex

To determine whether a $\alpha_L\tau\delta\delta'$ complex could form, the following components were mixed: α -large (400 µg, 2.5 nmol), τ (200 µg, 1.3 nmol), δ (200 µg,

4.8 nmol), δ' (200 μg, 5.75 pmol) in a final volume of 150 μl. The mixture was diluted to 300 ml with buffer A to lower conductivity of the sample to that equivalent of 100 mM NaCl and incubated for 30 min at 15°C. The mixture was injected onto a Superose 6 column (equilibrated with buffer A containing 100 mM NaCl) and fractions (170 μl) were collected after an initial 6.6 ml of void volume was collected. Fractions were analyzed by 10% SDS-polyacrylamide gels (30 μl/lane) stained with Coomassie Blue.

A gel filterable complex (Figure 18A) of $\alpha_L\tau\delta\delta'$ was formed as demonstrated by coellution of τ, δ and δ' with α -large (fr# 14-26), whereas excess δδ' complex elutes in later fractions (fr# 30-38). The migration of the τδδ' protein complex in the $\alpha_L\tau\delta\delta'$ complex does not change significantly. The complex might dissociate under the nonequilibrium conditions of gel filtration due to low concentration of proteins, salt concentration and speed of resolution.

Next, ion exchange chromatography was used to analyze the protein mixture to prepare the reconstituted $\alpha_L\tau\delta\delta'$ complex of *S. pyogenes*. The $\alpha_L\tau\delta\delta'$ complex was reconstituted upon mixing α -large (10 mg, 62 nmol), τ (6 mg, 72 nmol), δ (3.3 mg, 80 nmol), δ' (1.6 mg, 90 nmol). The α, τ, δ, δ' protein mixture was dialyzed for 2 hrs against buffer A containing 50 mM NaCl. The entire mixture was loaded onto a 1 ml MonoQ column equilibrated in buffer A containing 50 mM NaCl. Proteins were eluted with a 20 column volume linear gradient of 50-500 mM NaCl in buffer A and 0. 25 ml fractions were collected. Fractions were analyzed by 10% SDS-polyacrylamide gels (20 μl/lane) stained with Coomassie Blue.

Generally, the reconstitution of the $\alpha_L\tau\delta\delta'$ complex on a MonoQ column results in a tight salt resistant complex (Figure 18B, fr# 23-35) which elutes at 500 mM NaCl. The high concentration of the proteins in the eluted fractions contributes to stability of the complex.

Example 34 - The *S. pyogenes* Three Component Pol III-L Polymerase Is Rapid and Processive In DNA Synthesis

It was previously demonstrated (i.e., in Examples 29 and 30) that the putative delta subunit plays an integral part in the assembly of the τδδ' complex

(Figure 15) and that this complex is sufficient to assemble β clamps onto circular primed DNA (Figure 16). It was also shown that the strong interaction between the α - large and τ subunits (Figure 17) results in an isolatable $\alpha_L\tau\delta\delta'$ complex (Figure 18), similar to that of the *E. coli* DNA polymerase III*.

The MonoQ fractions containing $\alpha_L\tau\delta\delta'$ complex were then used to assemble β onto primed DNA and determine whether this now resulted in rapid and processive DNA synthesis. Replication reactions contained 70 ng of singly primed M13mp18 ssDNA and 0.82 μg of *S. pyogenes* SSB in 25 μl buffer C (20 mM Tris-HCl (pH 7.5), 4 % glycerol, 0.1 mM EDTA, 5 mM DTT, 2 mM ATP, 8 mM $MgCl_2$) with 60 μM each of dGTP, dCTP, and dATP, 30 μM cold TTP and 20 μM [α-32P] TTP (specific activity of 2,000-4,000 cpm/pmol). The complex is assembled onto DNA in the following manner: 40 ng (3:1) or 140 ng (10:1) of the $\alpha_L\tau\delta\delta'$ complex and 60 ng of β protein were preincubated for 2 min at 30°C in presence of SSB coated primed M13 DNA and two nucleotides (dCTP and dGTP). Reactions were initiated by addition of the two remaining nucleotides dATP and TTP and quenched with an equal volume of 1% SDS/40 mM EDTA. Each time point is a separate reaction.

A time course of replication on singly primed circular M13mp18 ssDNA is shown in Figure 19. The agarose gel analysis shows conversion of the oligonucleotide primed single stranded DNA to the slower migrating replicative form II. The fact that the speed of synthesis is independent of the concentration of polymerase in the reaction indicates that the $\alpha_L\tau\delta\delta'$ complex synthesizes DNA in a rapid and a highly processive manner. The *S. pyogenes* $\alpha_L\tau\delta\delta'$ complex in presence of the β clamp, completely replicates (is able to complete replication of) 7250 nt of M13mp18 ssDNA in 8-9 sec.

Example 35 - The S. pyogenes DnaE (α-small) Forms a Three-Component Polymerase with τδδ' and β

The *S. pyogenes* DnaE (α-small) polymerase is more homologous to *E. coli* α than *S. pyogenes* PolC. Thus, it seems reasonable to expect that the DnaE polymerase may also function with the β clamp (Figs. 21A-B). To test DnaE for function with τδδ' and β, replication reactions contained 70 ng (25 fmol) of 30-mer singly primed

M13mp18 ssDNA, 0.82 μg of *S. pyogenes* SSB, and 3.3 ng - 300 ng of DnaE (25 fmol - 2.3 pmol) in 23.5 μl of 20 mM Tris-HCl (pH 7.5), 4% glycerol, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 40 μg/ml BSA, 2 mM ATP, 8 mM $MgCl_2$, and 60 μM each of dGTP and dCTP. When present, reactions included 43.3 ng of β and 10 ng of τδδ'. Reactions were preincubated for 3 min at 37°C, and then NaCl was added to 40 mM followed by another 2 min at 37°C. DNA synthesis was initiated upon addition of 1.5 μl of 1.5 mM dATP, 0.5 mM [α^{32}P]-dTTP (specific activity 2,000-4,000 cpm/pmol). Aliquots of 25 μl were removed at the indicated times and quenched with an equal volume (25 μl) of 1% SDS, 40 mM EDTA. One-half of the quenched reaction was analyzed for total deoxynucleotide incorporation using DE81 filter paper and the other half was analyzed on a 0.8% neutral agarose gel. The effect of TMAU was also examined, in which 100μM TMAU in DMSO (2% DMSO final concentration) was present. In this case, replication was allowed to proceed for 1 min before being quenched with 25 μl of 1% SDS, 40 mM EDTA.

At a saturating concentration of DnaE polymerase, the time course of primer extension shows that it completes an M13mp18 primed ssDNA template within 2 minutes for a speed of at least 60 nucleotides/s (Fig. 21C). This rate of synthesis holds true for the highest amount of DnaE in the rightmost panel of the figure. As the DnaE concentration is decreased, a longer time is required to complete the circular template, indicating that the DnaE polymerase is not processive over the entire length of the M13mp18 template. If the DnaE polymerase were fully processive during synthesis of the 7.2 kb ssDNA circle, the product profile over time would be qualitatively similar at all concentrations of enzyme, but the overall intensity of the profile would be diminished. This particular experiment was performed in the absence of β, but presence of τδδ'. When repeated in the presence of β but without τδδ', and in the absence of both β and τδδ', results similar to those shown in Fig. 21C were observed.

In the presence of β and τδδ', DnaE polymerase is stimulated in synthesis at low concentration, indicating that β increases the processivity and/or speed of DnaE (Figs. 21C-D). At higher concentrations of DnaE, the presence of β/τδδ' has no effect on the rate of synthesis, and thus β does not increase the intrinsic speed of the enzyme (i.e., panels 3 and 4 of Fig. 21D). Hence, the effect of the β clamp on DnaE is

primarily due to an increase in processivity. The profile of product length over time remains essentially unchanged at the different DnaE concentrations, and therefore the processivity of DnaE, with β is at least equal to the 7.2 kb length of the M13mp18 substrate.

The DnaE sequence does not show homology to an exonuclease, implying that it may have no associated nuclease activity. The DnaE preparation was examined for the presence of a 3'-5' exonuclease (Fig. 21E). The DnaE and PolC polymerases were each incubated with a 5' 32P-labeled oligonucleotide, followed by analysis in a sequencing gel. The result showed no degradation of the oligonucleotide by DnaE. PolC is a known 3'-5' exonuclease and it digests the end-labeled oligonucleotide as expected.

Gram positive PolC is known to be inhibited by the antibiotic hydroxyphenylaza-uracil ("HPUra") and its derivatives. In Fig. 21F, the PolC·τδδ', β and DnaE were tested for inhibition of synthesis on SSB coated primed M13mp18 ssDNA by an HPUra derivative, trimethylanilino-uracil ("TMAU"). The PolC·τδδ' β enzyme was prevented from forming the RFII product by TMAU. In contrast, the DnaE polymerase was not affected by TMAU in the presence of τδδ'/β (nor in the absence of τδδ'/β, not shown).

Although the invention has been described in detail for the purpose of illustration, it is understood that such detail is solely for that purpose, and variations can be made therein by those skilled in the art without departing from the spirit and scope of the invention which is defined by the following claims.

WHAT IS CLAIMED:

1. An isolated DNA molecule from a Gram positive bacterium, the isolated DNA molecule comprising a coding region from a *polC* gene, a *dnaE* gene, a *holA* gene, a *holB* gene, a *dnaX* gene, a *dnaN* gene, a *ssb* gene, a *dnaG* gene, or a *dnaB* gene.

2. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *polC* gene.

3. The isolated DNA molecule according to claim 2, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

4. An isolated DNA molecule according to claim 3, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 18.

5. The isolated DNA molecule according to claim 4, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 17.

6. The isolated DNA molecule according to claim 2, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 17 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

7. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *dnaE* gene.

8. The isolated DNA molecule according to claim 7, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

9. The isolated DNA molecule according to claim 8, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 20.

10. The isolated DNA molecule according to claim 9, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 19.

11. The isolated DNA molecule according to claim 7, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 19 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

12. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *holA* gene.

13. The isolated DNA molecule according to claim 12, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

14. The isolated DNA molecule according to claim 13, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 22.

15. The isolated DNA molecule according to claim 14, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 21.

16. The isolated DNA molecule according to claim 12, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 21 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

17. The isolated DNA molecule according to claim 12, wherein the Gram positive bacterium is *Staphylococcus aureus*.

18. The isolated DNA molecule according to claim 17, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 12.

19. The isolated DNA molecule according to claim 18, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 11.

20. The isolated DNA molecule according to claim 12, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 11 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

21. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding regiong from the *holB* gene.

22. The isolated DNA molecule according to claim 21, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

23. The isolated DNA molecule according to claim 22, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 24.

24. The isolated DNA molecule according to claim 23, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 23.

25. The isolated DNA molecule according to claim 21, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 23 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

26. The isolated DNA molecule according to claim 21, wherein the Gram positive bacterium is *Staphylococcus aureus*.

27. The isolated DNA molecule according to claim 26, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 14.

28. The isolated DNA molecule according to claim 27, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 13.

29. The isolated DNA molecule according to claim 21, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 13 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

30. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *dnaX* gene.

31. The isolated DNA molecule according to claim 30, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

32. The isolated DNA molecule according to claim 31, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 26.

33. The isolated DNA molecule according to claim 32, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 25.

34. The isolated DNA molecule according to claim 30, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 25 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

35. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *dnaN* gene.

36. The isolated DNA molecule according to claim 35, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

37. The isolated DNA molecule according to claim 36, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 28.

38. The isolated DNA molecule according to claim 37, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 27.

39. The isolated DNA molecule according to claim 35, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 27 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

40. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *ssb* gene.

41. The isolated DNA molecule according to claim 40, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

42. The isolated DNA molecule according to claim 41, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 30.

43. The isolated DNA molecule according to claim 42, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 29.

44. The isolated DNA molecule according to claim 40, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 29 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

45. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *dnaG* gene.

46. The isolated DNA molecule according to claim 45, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

47. The isolated DNA molecule according to claim 46, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 32.

48. The isolated DNA molecule according to claim 47, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 31.

49. The isolated DNA molecule according to claim 45, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 31 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

50. The isolated DNA molecule according to claim 1, wherein the DNA molecule comprises the coding region from the *dnaB* gene.

51. The isolated DNA molecule according to claim 50, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

52. The isolated DNA molecule according to claim 51, wherein the DNA molecule encodes an amino acid sequence comprising SEQ. ID. No. 34.

53. The isolated DNA molecule according to claim 52, wherein the DNA molecule comprises a nucleotide sequence of SEQ. ID. No. 33.

54. The isolated DNA molecule according to claim 50, wherein the DNA molecule hybridizes to a nucleic acid molecule of SEQ. ID. No. 33 under stringent conditions characterized by use of a hybridization buffer comprising 0.9M SSC buffer at a temperature of 37°C.

55. An expression system comprising an expression vector into which is inserted a heterologous DNA molecule according to claim 1.

56. The expression system according to claim 55, wherein the heterologous DNA molecule is in sense orientation and correct reading frame.

57 A host cell comprising a heterologous DNA molecule according to claim 1.

58. An isolated protein or polypeptide from a Gram positive bacterium, wherein the isolated protein or polypeptide is alpha-large, alpha-small, delta, delta prime, tau, beta, SSB, DnaG, or DnaB.

59. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is alpha-large.

60. The isolated protein or polypeptide according to claim 59, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

61. The isolated protein or polypeptide according to claim 60, wherein the alpha-large protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 18.

62. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is alpha-small.

63. The isolated protein or polypeptide according to claim 62, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

64. The isolated protein or polypeptide according to claim 63, wherein the alpha-small protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 20.

65. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is delta.

66. The isolated protein or polypeptide according to claim 65, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

67. The isolated protein or polypeptide according to claim 66, wherein the delta protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 22.

68. The isolated protein or polypeptide according to claim 65, wherein the Gram positive bacterium is *Staphylococcus aureus*.

69. The isolated protein or polypeptide according to claim 68, wherein the delta protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 12.

70. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is delta prime.

71. The isolated protein or polypeptide according to claim 70, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

72. The isolated protein or polypeptide according to claim 71, wherein the delta prime protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 24.

73. The isolated protein or polypeptide according to claim 70, wherein the Gram positive bacterium is *Staphylococcus aureus*.

74. The isolated protein or polypeptide according to claim 73, wherein the delta prime protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 14.

75. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is tau.

76. The isolated protein or polypeptide according to claim 75, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

77. The isolated protein or polypeptide according to claim 76, wherein the tau protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 26.

78. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is beta.

79. The isolated protein or polypeptide according to claim 78, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

80. The isolated protein or polypeptide according to claim 79, wherein the beta protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 28.

81. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is SSB.

82. The isolated protein or polypeptide according to claim 81, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

83. The isolated protein or polypeptide according to claim 82, wherein SSB comprises an amino acid sequence of SEQ. ID. No. 30.

84. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is DnaG.

85. The isolated protein or polypeptide according to claim 84, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

86. The isolated protein or polypeptide according to claim 85, wherein the DnaG protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 32.

87. The isolated protein or polypeptide according to claim 58, wherein the isolated protein or polypeptide is DnaB.

88. The isolated protein or polypeptide according to claim 87, wherein the Gram positive bacterium is *Streptococcus pyogenes*.

89. The isolated protein or polypeptide according to claim 88, wherein the DnaB protein or polypeptide comprises an amino acid sequence of SEQ. ID. No. 34.

90. A method of identifying compounds which inhibit the activity of a polymerase product of *polC* or *dnaE* comprising:

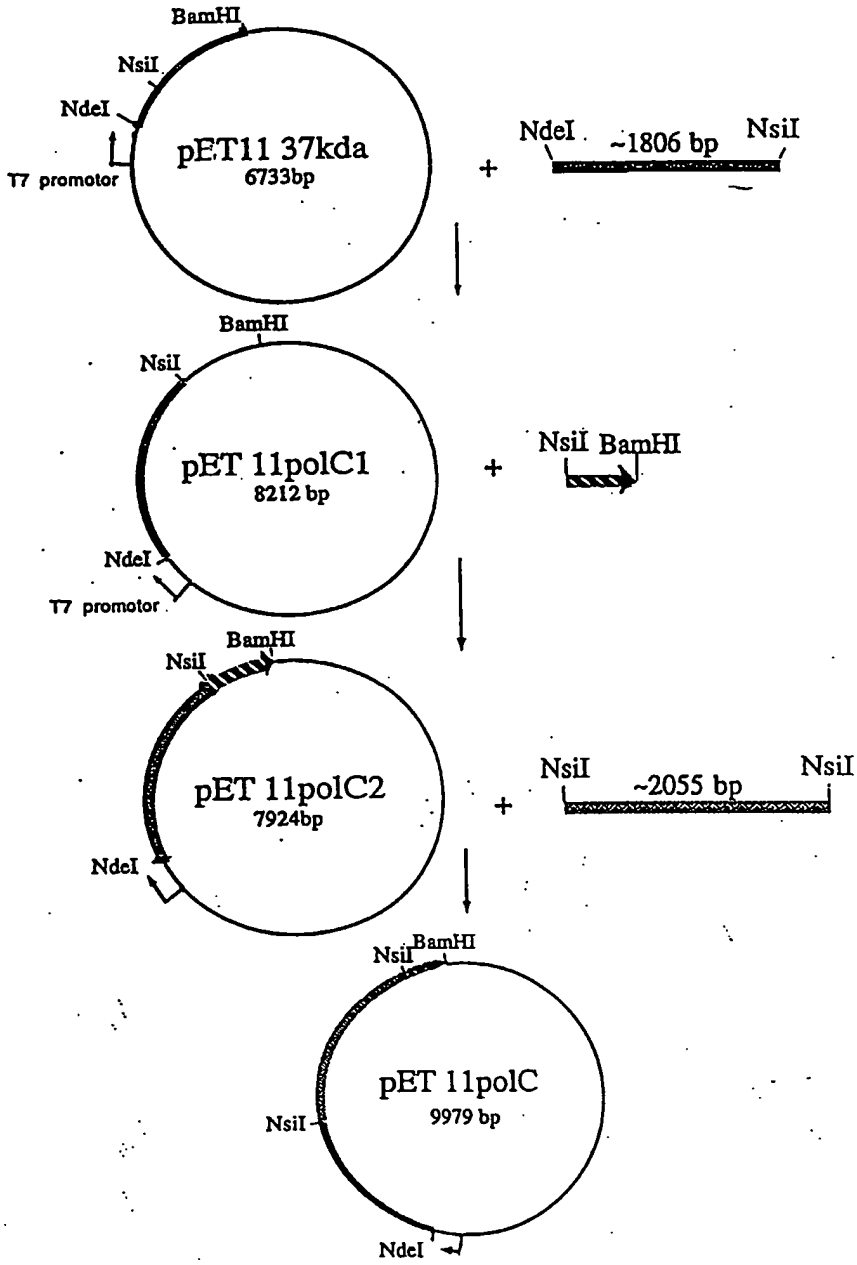
forming a reaction mixture comprising a primed DNA molecule, a polymerase product of *polC* or *dnaE*, a candidate compound, a dNTP, and optionally either a beta subunit, a tau complex, or both the beta subunit and the tau complex, wherein at least one of the polymerase product of *polC* or *dnaE*, the beta subunit, the tau complex, or a subunit or combination of subunits thereof is derived from a Eubacteria other than *Escherichia coli*;

subjecting the reaction mixture to conditions effective to achieve nucleic acid polymerization in the absence of the candidate compound;

analyzing the reaction mixture for the presence or absence of nucleic acid polymerization extension products; and

identifying the candidate compound in the reaction mixture where there is an absence of nucleic acid polymerization extension products.

91. The method according to claim 90, wherein the polymerase product of *polC* or *dnaE* is from a *Streptococcus* bacterium or a *Staphylococcus* bacterium.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NsiI
NdeI
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6733bp
T7 promotor
NdeI ~1806 bp NsiI
BamHI
NsiI
pET 11polC1
8212 bp
NdeI
T7 promotor
NsiI BamHI
BamHI
NsiI
pET 11polC2
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NsiI
BamHI
pET 11polC
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NsiI
NdeI

FIGURE 1

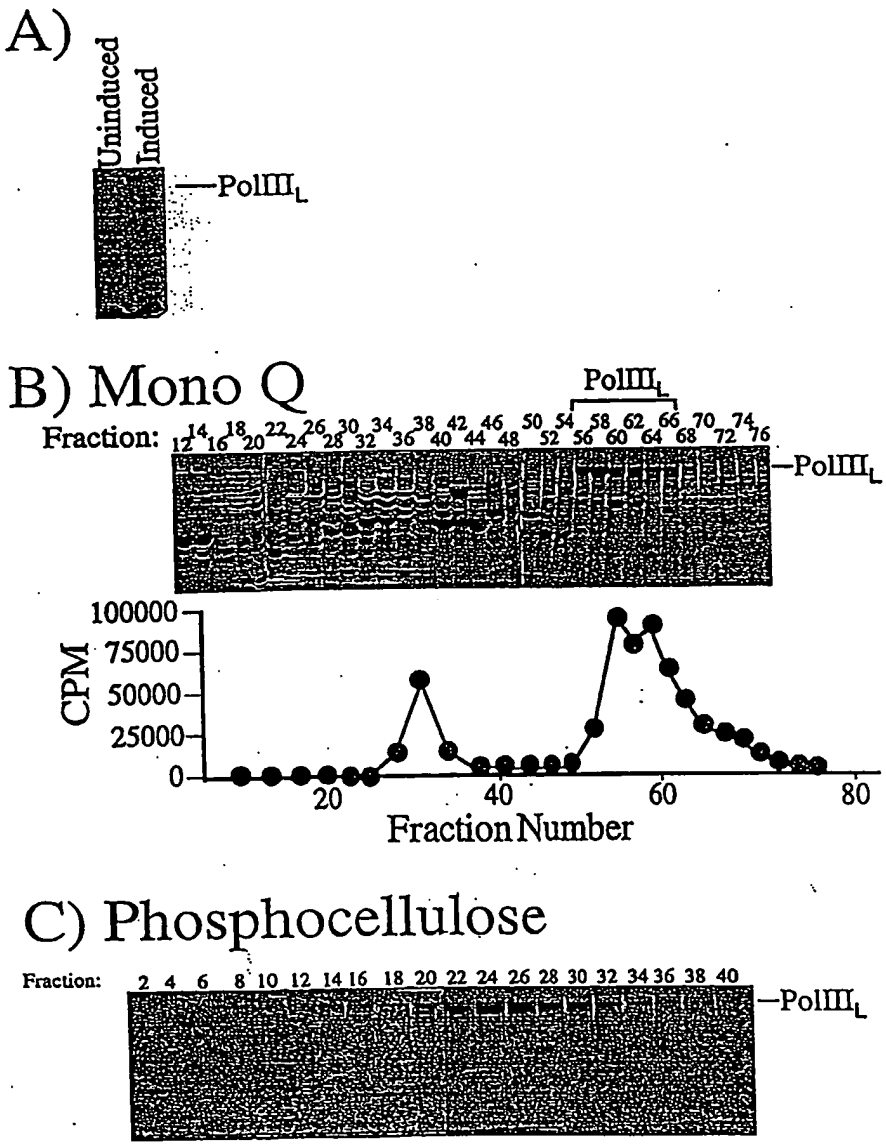

A)
Uninduced
Induced
PolIII$_L$
B) Mono Q
PolIII$_L$
Fraction: 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76
PolIII$_L$
CPM
100000
75000
50000
25000
0
20
40
60
80
Fraction Number
C) Phosphocellulose
Fraction: 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
PolIII$_L$

FIGURE 2

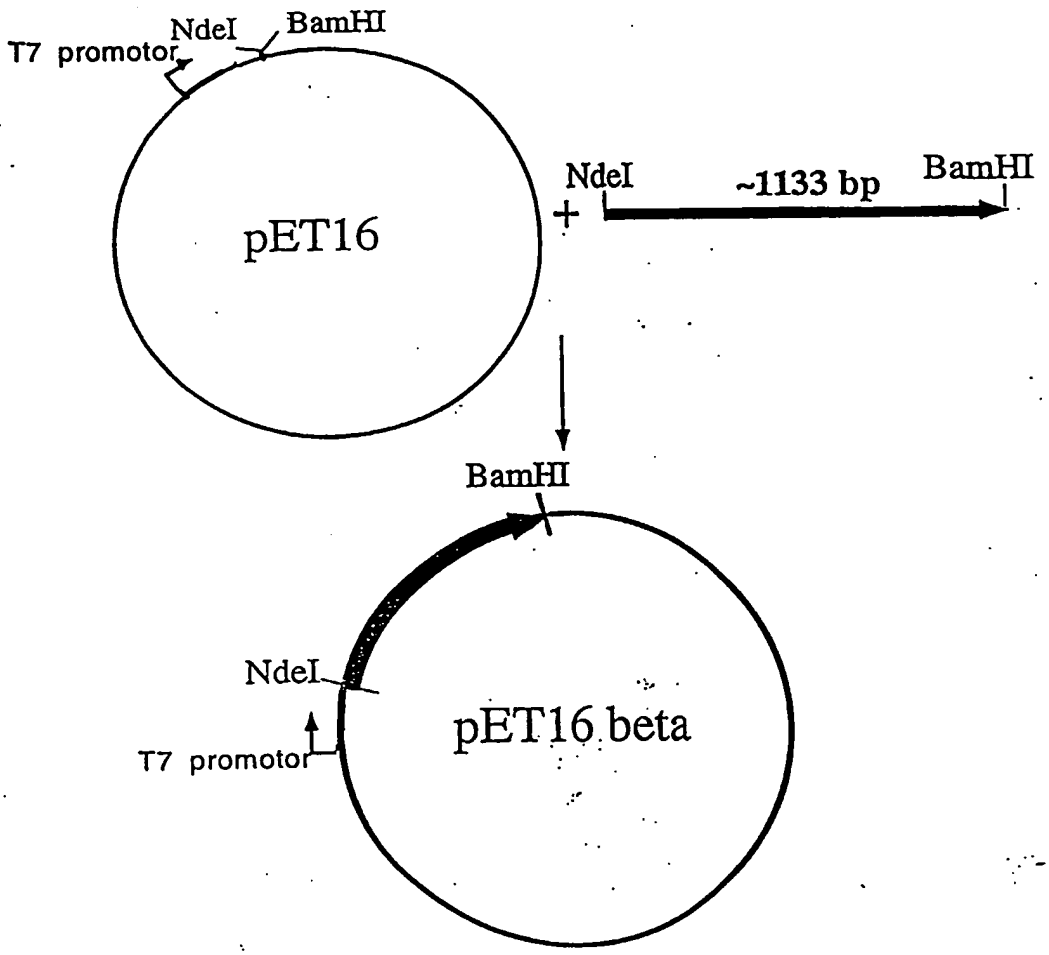

NdeI
BamHI
T7 promotor
pET16
NdeI
~1133 bp
BamHI
BamHI
NdeI
T7 promotor
pET16 beta

FIGURE 3

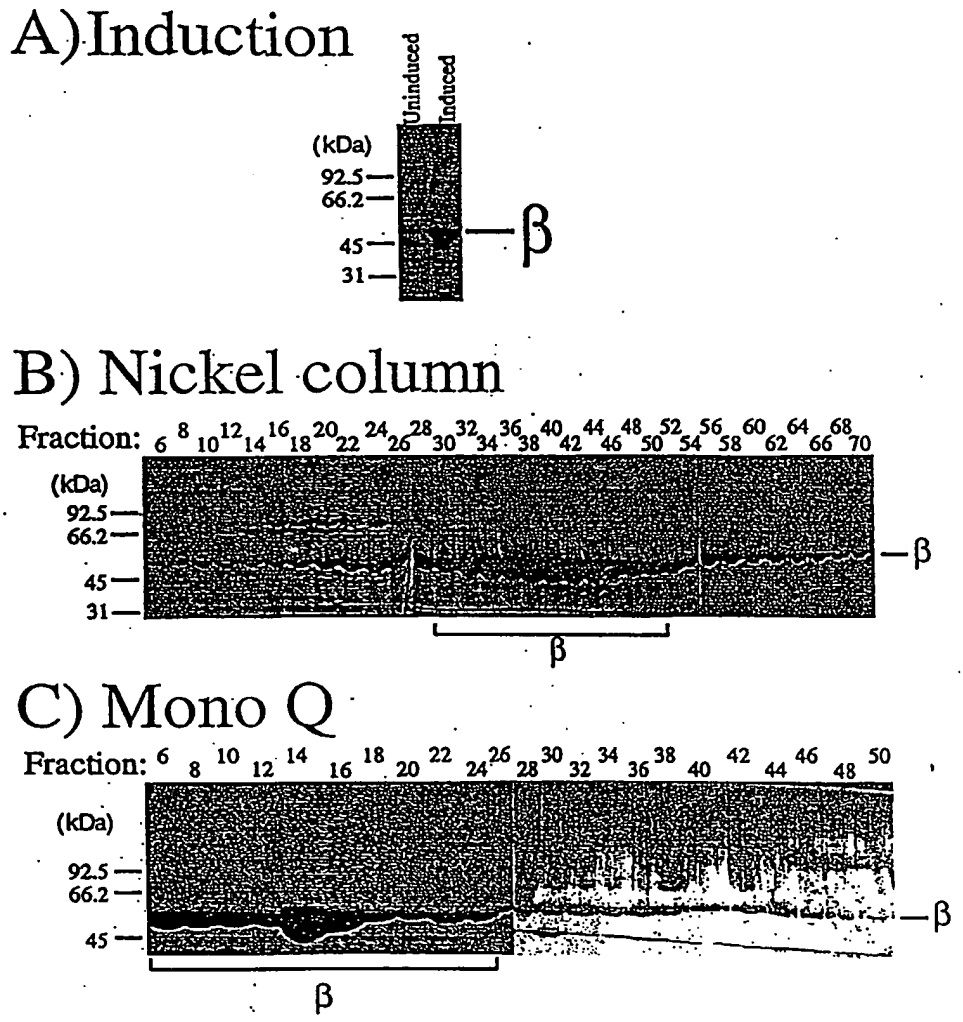
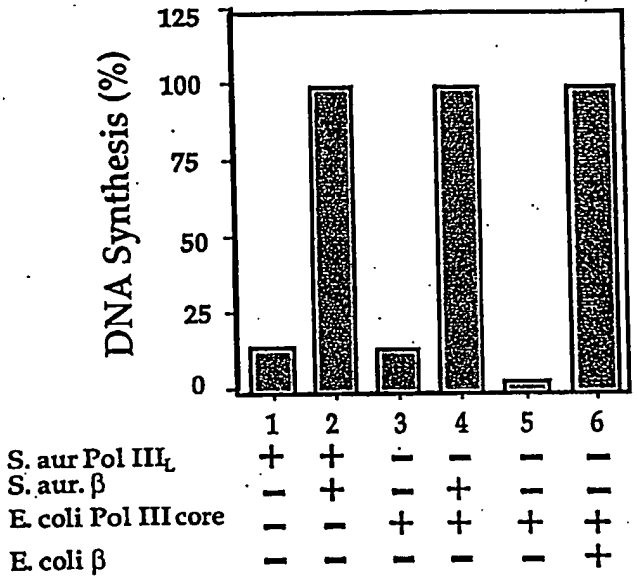

A) Induction
Uninduced
Induced
(kDa)
92.5
66.2
45
31
β
B) Nickel column
Fraction: 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70
(kDa)
92.5
66.2
45
31
β
β
C) Mono Q
Fraction: 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
(kDa)
92.5
66.2
45
β
β

FIGURE 4

A) Linear DNA



B) Circular DNA

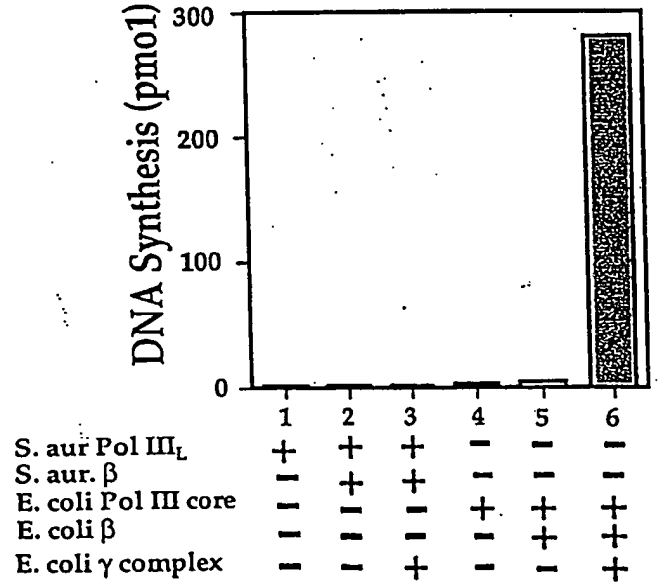


FIGURE 5

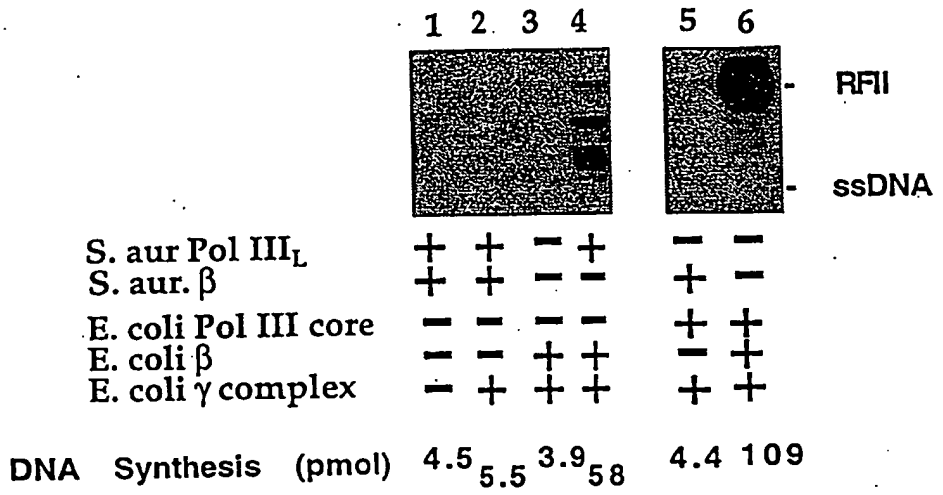

1 2 3 4 5 6
RFII
ssDNA
S. aur Pol III$_L$ + + − + − −
S. aur. β + + − − + −
E. coli Pol III core − − − − + +
E. coli β − − + + − +
E. coli γ complex − + + + + +
DNA Synthesis (pmol) 4.5 5.5 3.9 58 4.4 109

FIGURE 6

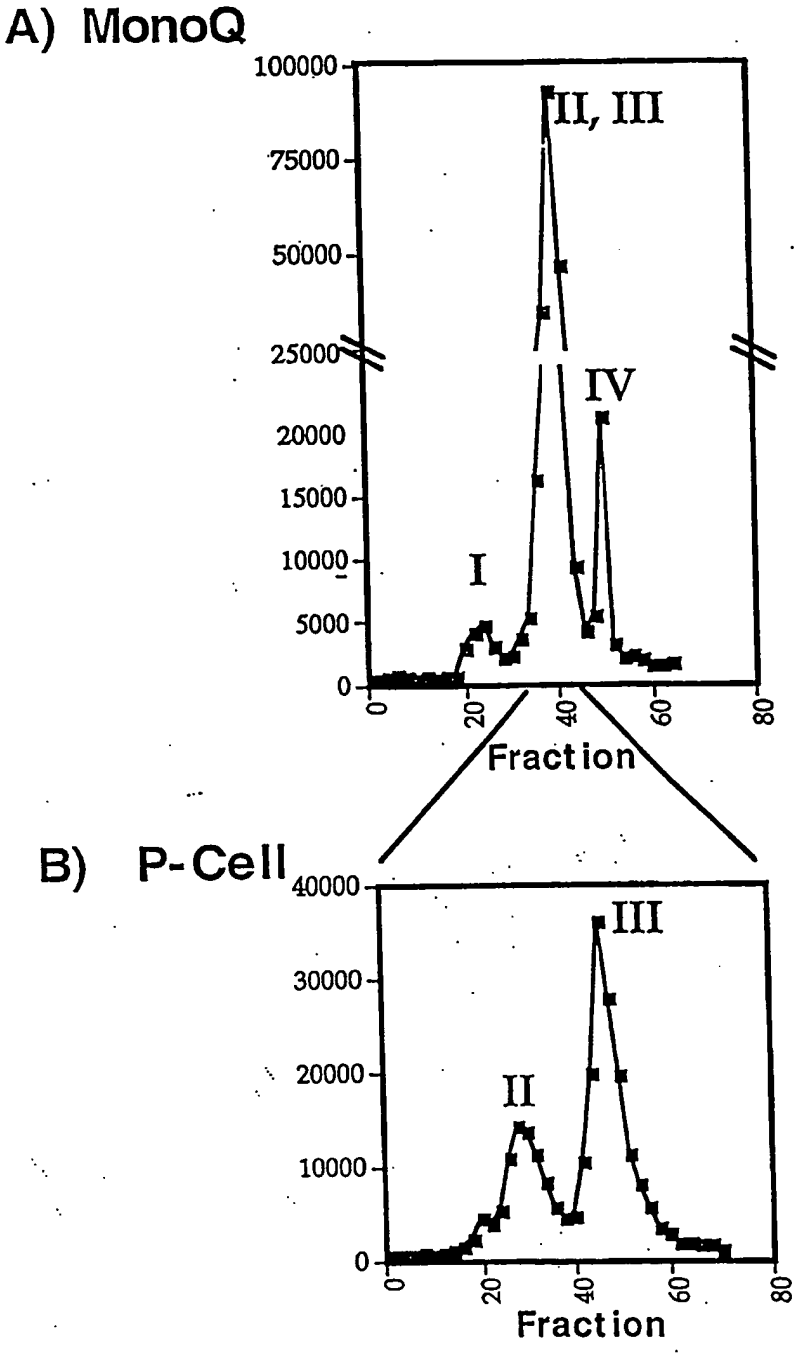
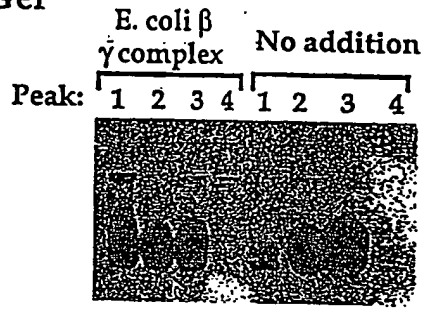

A) MonoQ
100000
75000
50000
25000
20000
15000
10000
5000
0
II, III
IV
I
0
20
40
60
80
Fraction
B) P-Cell
40000
30000
20000
10000
0
III
II
0
20
40
60
80
Fraction

FIGURE 7

A) Agarose Gel



B) DNA Synthesis

	DNA Synthesis (pmol) PEAK			
Addition	Peak 1	Peak 2	Peak 3	Peak 4
None	22.7	70.6	146.1	4.7
E. coli β, γ complex	72.9	61.2	71.4	25.9

FIGURE 8

```
S.aureus    KIWRATCIWNCDFRSSACKAVAKDVGRIMGFDEVTLNEISSLIPHKLGITLDEAYQID-D
E.coli      MYGRDAVSQIITFGTMAAKAVIRDVGRVLGHPYGFVDRISKLIPPDPGMTLAKAFEAEPQ
Sal.typ     MYGRDAVSQIITFGTMAAKAVIRDVGRVLGHPYGFVDRISKLVPPDPGMTLAKAFEAEPQ
                        * . *.*** .****..*   .   ** *.*   *.**  *.. .

S.aureus    FKKFVHRNHRHQRWFSICKKLEGLPRHTSTHAAGIIINDHPLYEYAPLTKGDTG--LLTQ
E.coli      LPEIYEADEEVKALIDMARKLEGVTRNAGKHAGGVVIAPTKITDFAPLYCDEEGKHPVTQ
Sal.typ     LPEIYEADEEVRALIDMARKLEGVTRNAGKHAGGVVIAPTKITDFAPLYCDEEGKHPVTQ
                .     ...****. *..  ** *..*    . ..***    . *   .**

S.aureus    WTMTEAERIGLLKIDFLGLRNLSIIHQILTRVEKDLGFN----IDIEKIPFDDQKVFELL
E.coli      FDKSDVEYAGLVKFDFLGLRTLTIINWALEMINKRRAKNGEPPLDIAAIPLDDKKSFDML
Sal.typ     FDKSDVEYAGLVKFDFLGLRTLTIINWALEMINKRRAKNGEPPLDIAAIPLDDKKSFDML
              .. *  **.* ******.*.**.  *  . *     *    .**  ** **.* *..*

S.aureus    SQGDTTGIFQLESDGVRSVLKKLKPEHFEDIVAVTSLYRPGPMEE--IPTYITRRHDPS-
E.coli      QRSETTAVFQLESRGMKDLIKRLQPDCFEDMIALVALFRPGPLQSGMVDNFIDRKHGREE
Sal.typ     QRSETTAVFQLESRGMKDLIKRLQPDCFEDMIALVALFRPGPLQSGMVDNFIDRKHGREE
             . .** .***** *.. ..*.*.*. ***..*. .*.****..    . ..*.*.*

S.aureus    ----KVQYLHPHLEPILKNTYGVIIYQEQIMQIASTFANFSYGEADILRRAMSKKNRAVL
E.coli      ISYPDVQWQHESLKPVLEPTYGIILYQEQVMQIAQVLSGYTLGGADMLRRAMGKKKPEEM
Sal.typ     LSYPDVQWQHESLKPVLEPTYGIILYQEQVMQIAQVLSGYTLGGADMLRRAMGKKKPEEM
                 **  *  * *.*  ***.*.****.****    .... * **.***** **    .

S.aureus    ERDAQHFIEGTKQNGYHEDISKQIFDLI--------------------------------
E.coli      AKQRSVFAEGAEKNGINAELAMKIFDLVEKFAGYGFNKSHSAAYALVSYQTLWLKAHYPA
Sal.typ     AKQRSVFEEGAKKNGIDGELAMKIFDLVEKFAGYGFNKSHSAAYALVSYQTLWLKAHYPA
             .     * **. .**   ... .****.
```

FIGURE 9

```
                                                                    ATP site
S.aureusMKGYCLWRCNLDYQALFVVPTP-KFEDVVGQEHSEDCAMG-------SHAYLFSGPRGTGKT
B.sub.  ----------MSYQALYRVFRPQRFEDVVGQEHITKTLQNALLQKKFSHAYLFSGPRGTGKT
E.coli  ----------MSYQVLARKWRPQTFADVVGQEHVLTALANGLSLGRIHHAYLFSGTRGVGKT
                    ** *     *  * *******  .  .        ******* ** ***

                 Zn++ finger
                   |        |  |  |
S.aureusSIAKVFAKAINCLNSTDGEPCNECHICKGITQGTNSDVIEIDAASNNGVDEIRNIRDKVKYA
B. sub  SAAKIFAKAVNCEHAPVDEPCNECAACKGITNGSISDVIEIDAASNNGVDEIRDIRDKVKFA
E. coli SIARLLAKGLNCETGITATPCGVCDNCREIEQGRFVDLIEIDAASRTKVEDTRDLLDNVQYA
         * *.. ** .**        **. *  *. * .*  *.******* . *.. * . * *..*

S.aureusPSESKYKVYIIDEVHMLTTGAFNALLKTLEEPPAHAIFILATTEPHKIPPTIISRA
B. sub  PSAVTYKVYIIDEVHMLSIGAFNALLKTLEEPPEHCIFILATTEPHKIPLTIISRC
E. coli PARGRFKVYLIDEVHMLSRHSFNALLKTLEEPPEHVKFLLATTDPQKLPVTILSRC
         *.   .***.*******.  .************ *  *.****.*.*.* **.**.
```

FIGURE 10

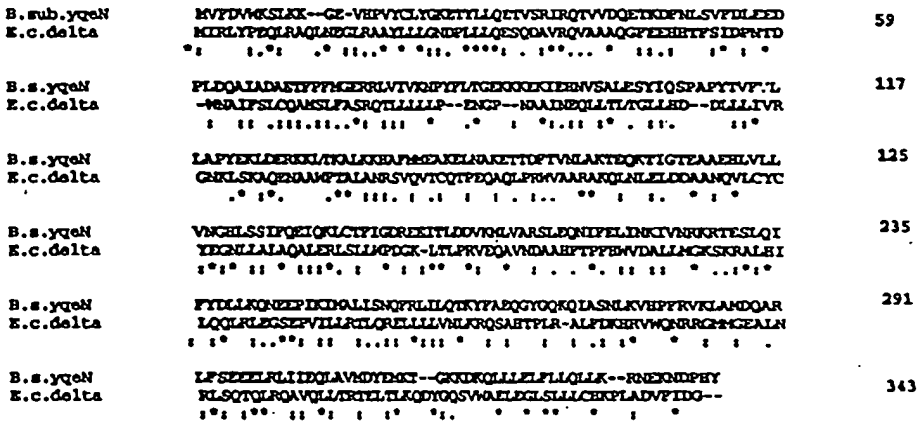
```
S.aureus  ALNIANKLERMKIYLAVGIFSLEMGADQLTTRMICSSGNVDSNRLRTGTMTEEDWSRFTI
B.sub     ALNIAQNVA-TKTDFSVAIFSLFMGAEQLVMRMLCAEGNINAQNLRTGNLTEEDWGKLTM
E.coli    AMNLVENAA-MLQDKPVLIFSLEMPSEQIMMRSLASLSRVDQTKIRTGQLDDEDWARISG
Sal.typ   AMNLCENAA-MLQDKPVLIFSLEMPGEQIMMRMLASLSRVDQTRIRTGQLDDEDWARISG
          *.*.      * **** * .*.  * ...  .  .*** . .*** . .

S.aureus  AVGKLS-RTKIFIDDTPGIPINDLRSKCRRLKQEHG-LYVIVIDYLQLIPGVGSRASDNR
B.sub     AMGSLS-NSGIYIDDIPGIRVSEIRAKCRRLKQESG-LGMILIDYLQLIQGSG-RSKDNR
E.coli    TMGILLEKRNIYIDDSSGLTPTEVRSRARRIAREHGGIGLIMIDYLQLMRVPA--LSDNR
Sal.typ   TMGILLEKRNMYIDDSSGLTPTEVRSRARRIFREHGGLSLIMIDYLQLMRVPS--LSDNR
          ..* *     ..*** *.   ..*.... **. . * * .  . *.******.     ***

S.aureus  QQEVSEISRTLKALARELECPVIADSQLSPALPPRRATRPDLPRH----------------
B.sub     QQEVSEISRELKSIARELQVPVIALSQLSRGVEQRQDKRPMMSDIRESGSIEQDADIVAF
E.coli    TLEIAEISRSLKALAKELNVPVVALSQLNRSLEQRADKRPVNSDLRESGSIEQDADLIMF
Sal.typ   TLEIAEISRSLKALAKELQVPVVALSQLNRSLEQRADKRPVNSDLRESGSIEQDADLIMF
            *..***** **..*.**  **.* ***    .  *   **
```

FIGURE 11

A



B

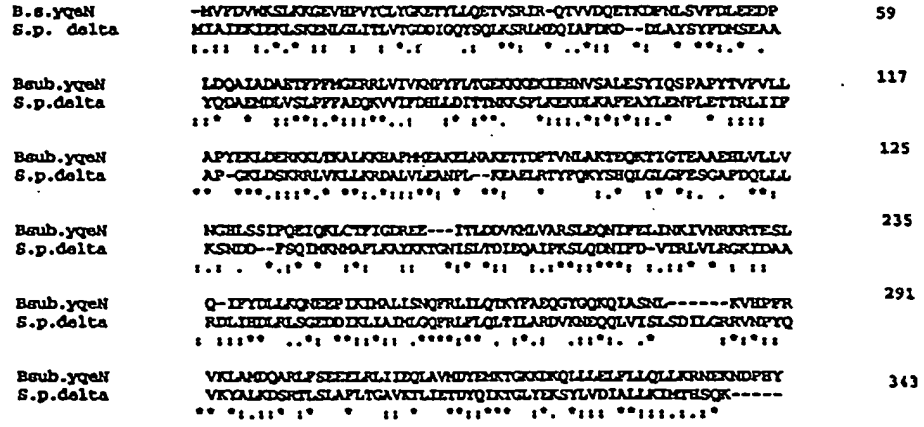


FIGURE 12

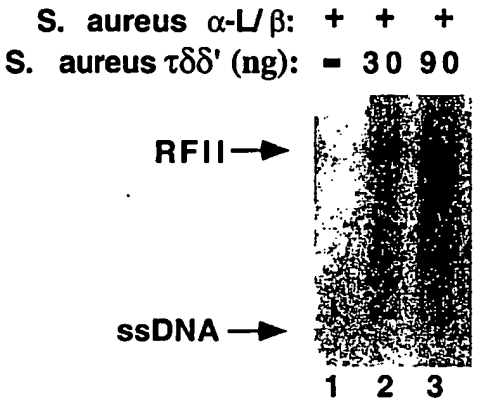


FIGURE 13

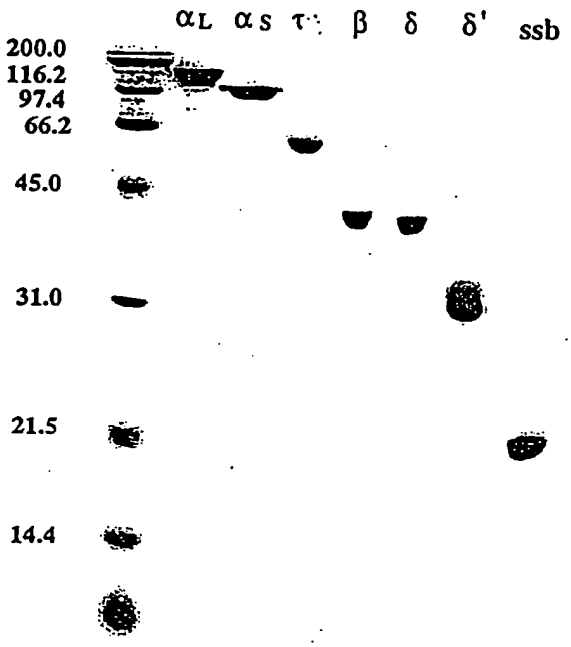
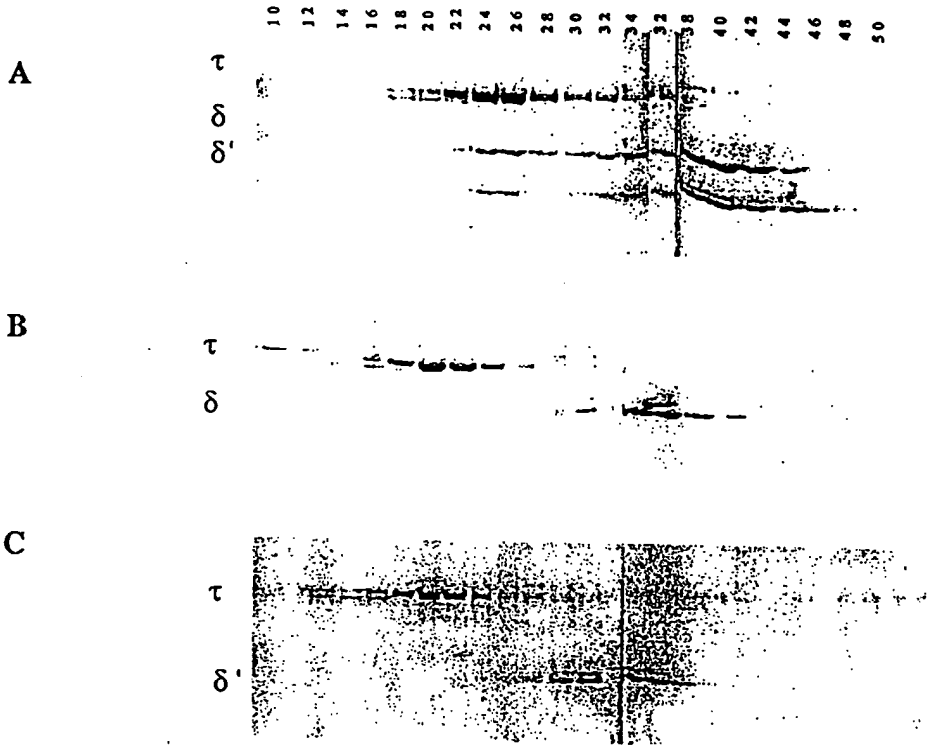


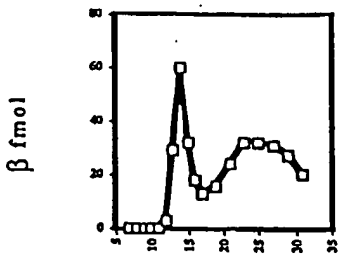
FIGURE 14

Superose 6



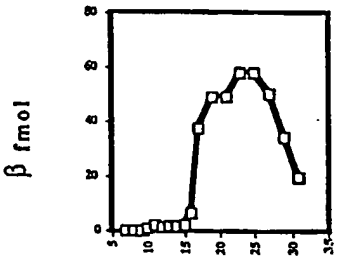
FIGURES 15A-C

A



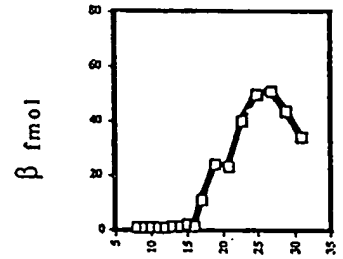
τδδ +β

B



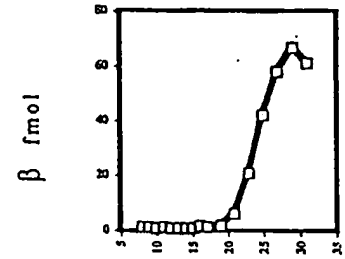
β alone

C



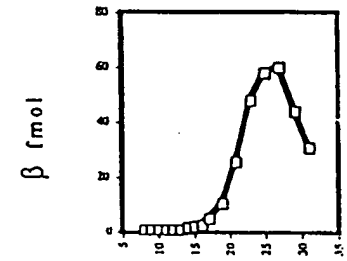
δδ' + β

D



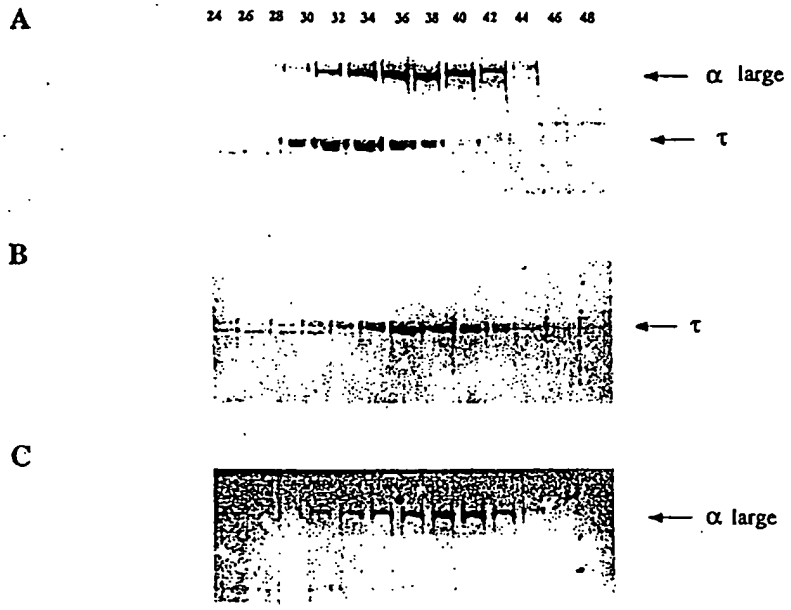
τδ+β

E



τδ' +β

FIGURES 16A-E



FIGURES 17A-C

Superose 6

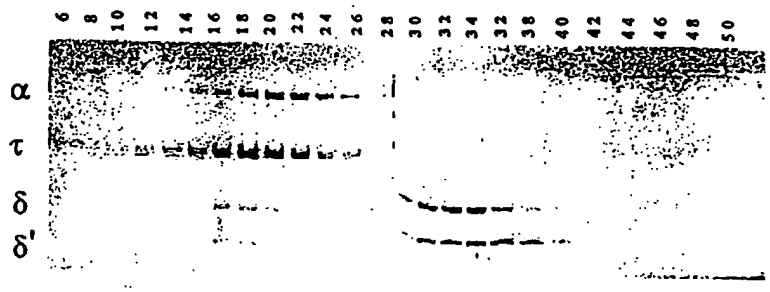


FIGURE 18

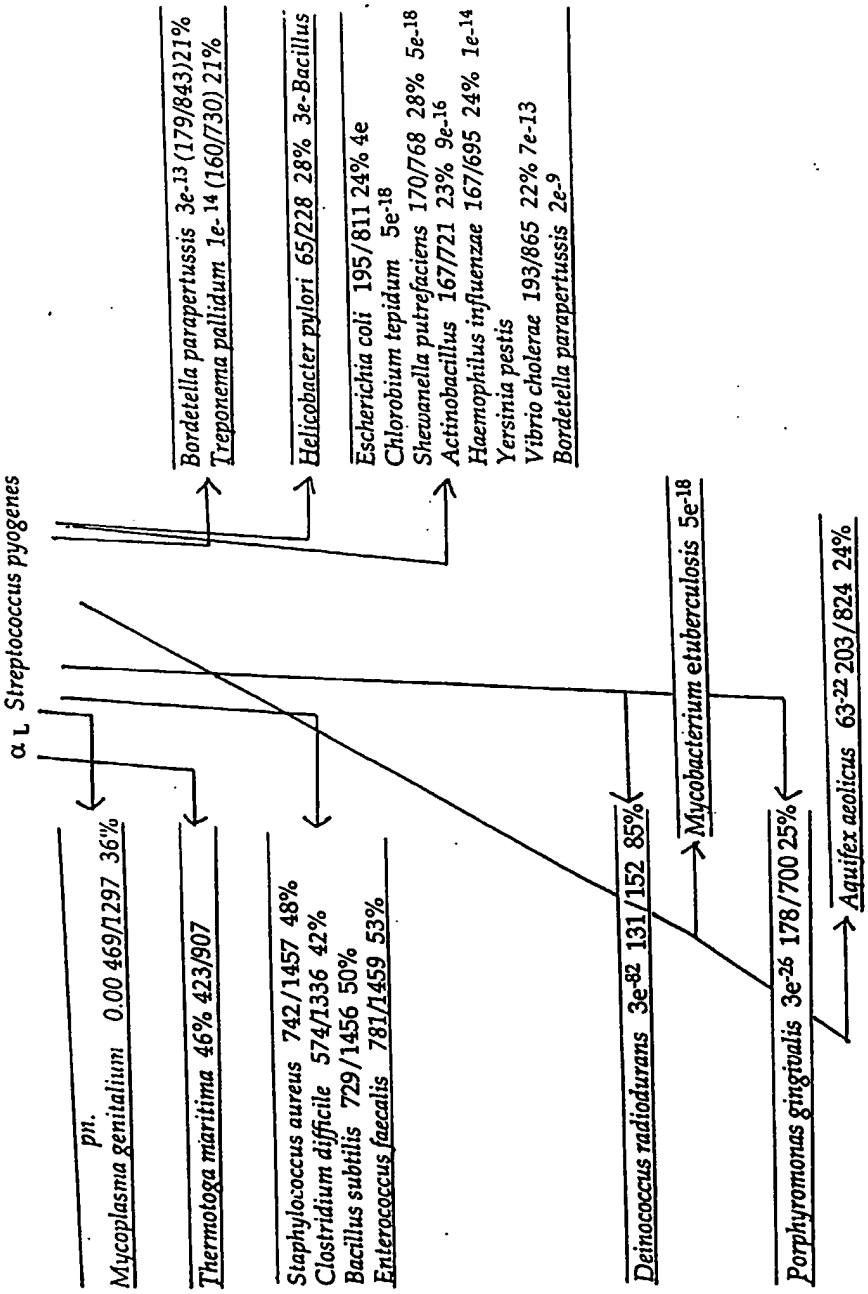

α L Streptococcus pyogenes
pn.
Mycoplasma genitalium 0.00 469/1297 36%
Thermotoga maritima 46% 423/907
Staphylococcus aureus 742/1457 48%
Clostridium difficile 574/1336 42%
Bacillus subtilis 729/1456 50%
Enterococcus faecalis 781/1459 53%
Deinococcus radiodurans 3e-82 131/152 85%
Porphyromonas gingivalis 3e-26 178/700 25%
Bordetella parapertussis 3e-13 (179/843)21%
Treponema pallidum 1e-14 (160/730) 21%
Helicobacter pylori 65/228 28% 3e-Bacillus
Escherichia coli 195/811 24% 4e
Chlorobium tepidum 5e-18
Shewanella putrefaciens 170/768 28% 5e-18
Actinobacillus 167/721 23% 9e-16
Haemophilus influenzae 167/695 24% 1e-14
Yersinia pestis
Vibrio cholerae 193/865 22% 7e-13
Bordetella parapertussis 2e-9
Mycobacterium etuberculosis 5e-18
Aquifex aeolicus 63-22 203/824 24%

FIGURE 20A

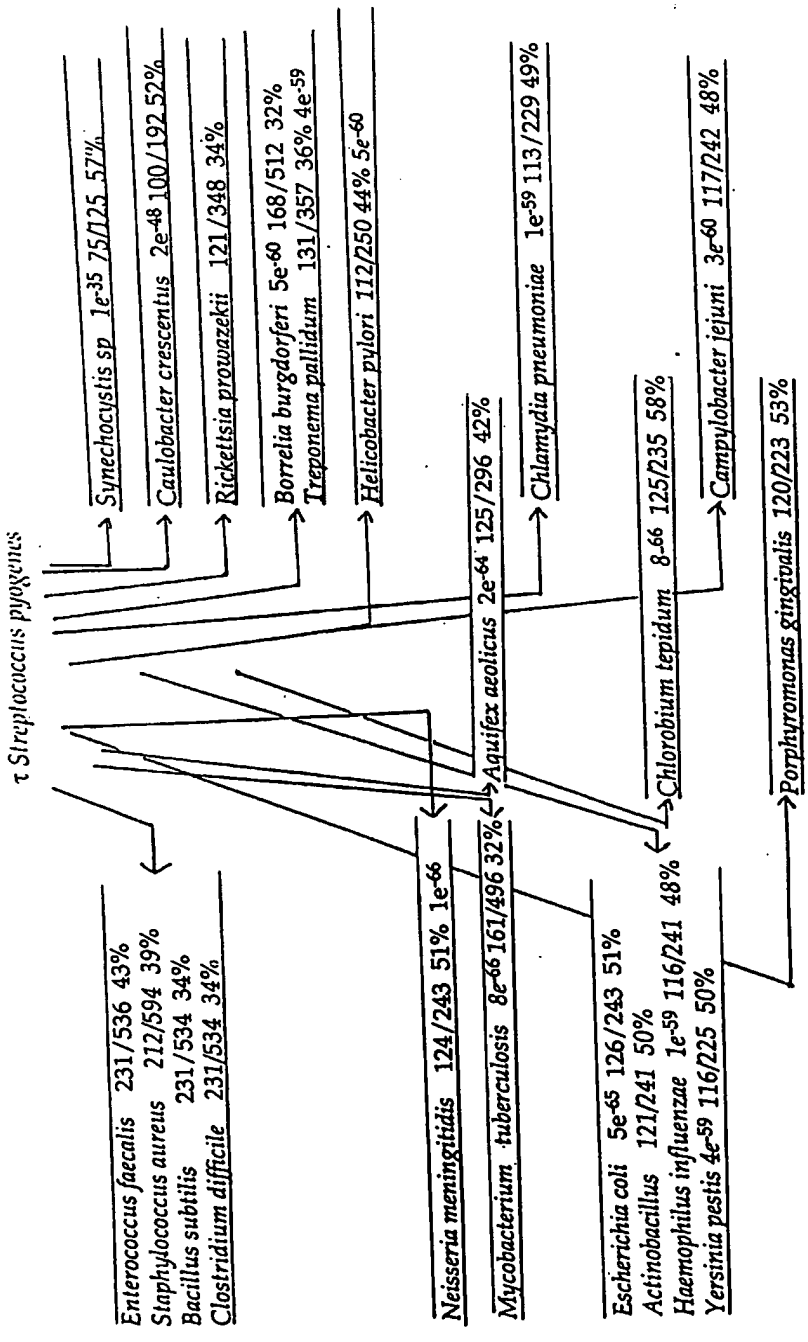

τ Streptococcus pyogenus
Enterococcus faecalis 231/536 43%
Staphylococcus aureus 212/594 39%
Bacillus subtilis 231/534 34%
Clostridium difficile 231/534 34%
Synechocystis sp 1e-35 75/125 57%
Caulobacter crescentus 2e-48 100/192 52%
Rickettsia prowazekii 121/348 34%
Borrelia burgdorferi 5e-60 168/512 32%
Treponema pallidum 131/357 36% 4e-59
Helicobacter pylori 112/250 44% 5e-60
Neisseria meningitidis 124/243 51% 1e-66
Aquifex aeolicus 2e-64 125/296 42%
Mycobacterium tuberculosis 8e-66 161/496 32%
Chlamydia pneumoniae 1e-59 113/229 49%
Escherichia coli 5e-65 126/243 51%
Actinobacillus 121/241 50%
Haemophilus influenzae 1e-59 116/241 48%
Yersinia pestis 4e-59 116/225 50%
Chlorobium tepidum 8-66 125/235 58%
Campylobacter jejuni 3e-60 117/242 48%
Porphyromonas gingivalis 120/223 53%

FIGURE 20B

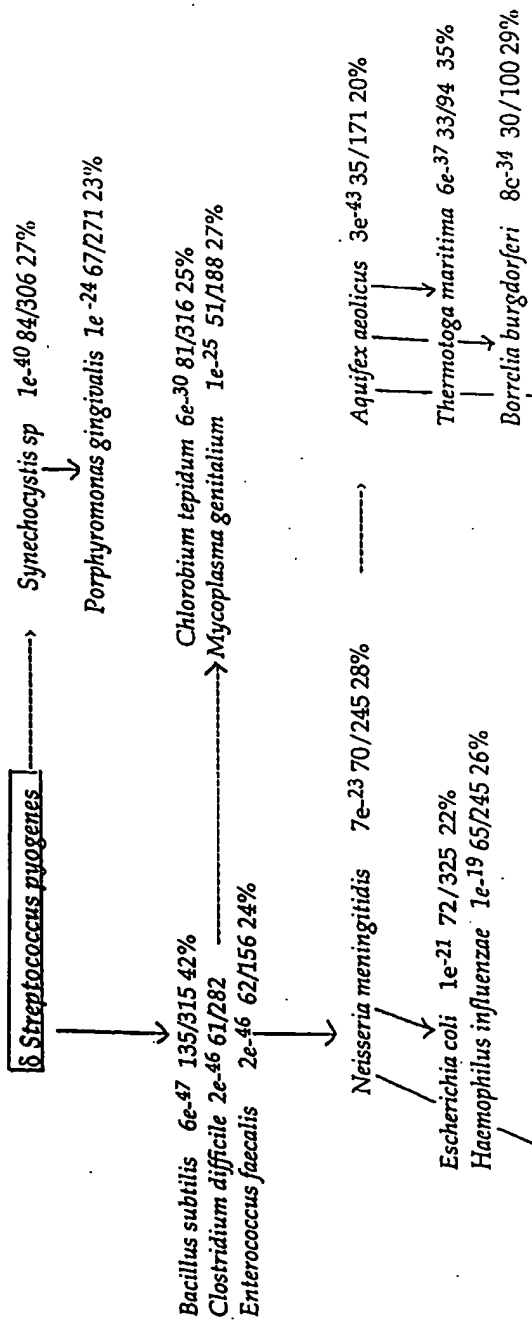

δ Streptococcus pyogenes
Synechocystis sp 1e-40 84/306 27%
Porphyromonas gingivalis 1e-24 67/271 23%
Chlorobium tepidum 6e-30 81/316 25%
Mycoplasma genitalium 1e-25 51/188 27%
Bacillus subtilis 6e-47 135/315 42%
Clostridium difficile 2e-46 61/282
Enterococcus faecalis 2e-46 62/156 24%
Neisseria meningitidis 7e-23 70/245 28%
Escherichia coli 1e-21 72/325 22%
Haemophilus influenzae 1e-19 65/245 26%
Thiobacillus ferrooxidans 3e-22 67/248 27%
Helicobacter pylori 4e-33 990/226 39%
Campylobacter jejuni 2e-35 46/206 22%
Aquifex aeolicus 3e-43 35/171 20%
Thermotoga maritima 6e-37 33/94 35%
Borrelia burgdorferi 8e-34 30/100 29%
Rickettsia prowazekii 1e-48
cout 1 36/154 23%
cout 2 13/49 49%

FIGURE 20D

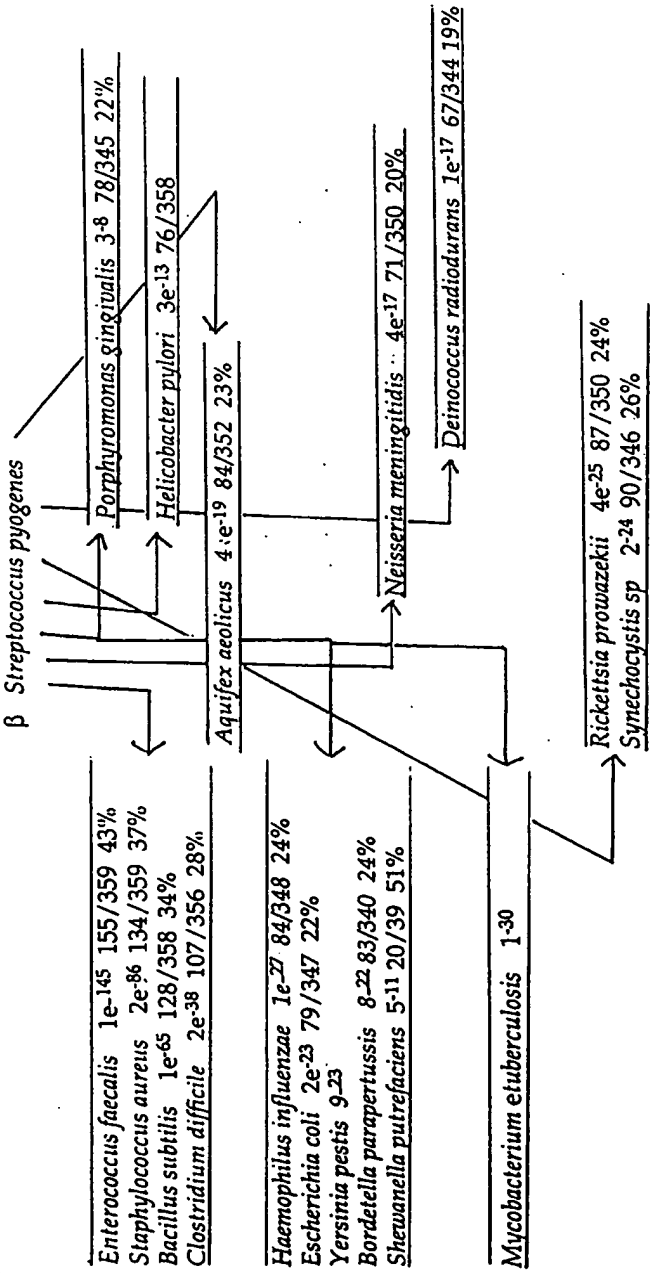

β Streptococcus pyogenes
Porphyromonas gingivalis 3-8 78/345 22%
Helicobacter pylori 3e-13 76/358
Aquifex aeolicus 4.e-19 84/352 23%
Neisseria meningitidis 4e-17 71/350 20%
Deinococcus radiodurans 1e-17 67/344 19%
Rickettsia prowazekii 4e-25 87/350 24%
Synechocystis sp 2-24 90/346 26%
Enterococcus faecalis 1e-145 155/359 43%
Staphylococcus aureus 2e-86 134/359 37%
Bacillus subtilis 1e-65 128/358 34%
Clostridium difficile 2e-38 107/356 28%
Haemophilus influenzae 1e-27 84/348 24%
Escherichia coli 2e-23 79/347 22%
Yersinia pestis 9-23
Bordetella parapertussis 8-22 83/340 24%
Shewanella putrefaciens 5-11 20/39 51%
Mycobacterium etuberculosis 1-30

FIGURE 20E


14%

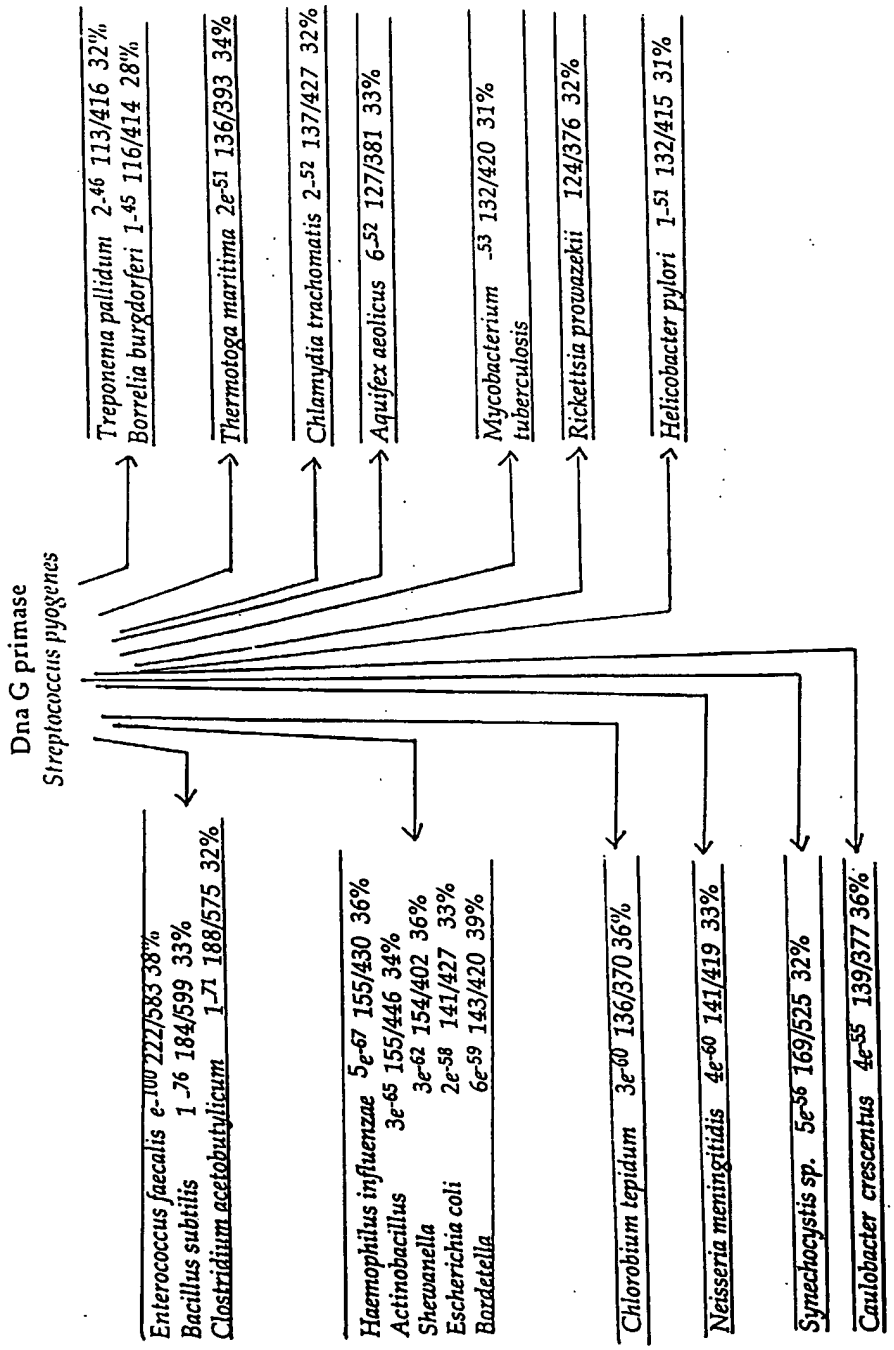

Dna G primase
Streptococcus pyogenes
Treponema pallidum 2-46 113/416 32%
Borrelia burgdorferi 1-45 116/414 28%
Thermotoga maritima 2e-51 136/393 34%
Chlamydia trachomatis 2-52 137/427 32%
Aquifex aeolicus 6-52 127/381 33%
Mycobacterium -53 132/420 31%
tuberculosis
Rickettsia prowazekii 124/376 32%
Helicobacter pylori 1-51 132/415 31%
Enterococcus faecalis e-100 222/583 38%
Bacillus subtilis 1-76 184/599 33%
Clostridium acetobutylicum 1-71 188/575 32%
Haemophilus influenzae 5e-67 155/430 36%
Actinobacillus 3e-65 155/446 34%
Shewanella 3e-62 154/402 36%
Escherichia coli 2e-58 141/427 33%
Bacteroides 6e-59 143/420 39%
Chlorobium tepidum 3e-60 136/370 36%
Neisseria meningitidis 4e-60 141/419 33%
Synechocystis sp. 5e-56 169/525 32%
Caulobacter crescentus 4e-55 139/377 36%

FIGURE 20G

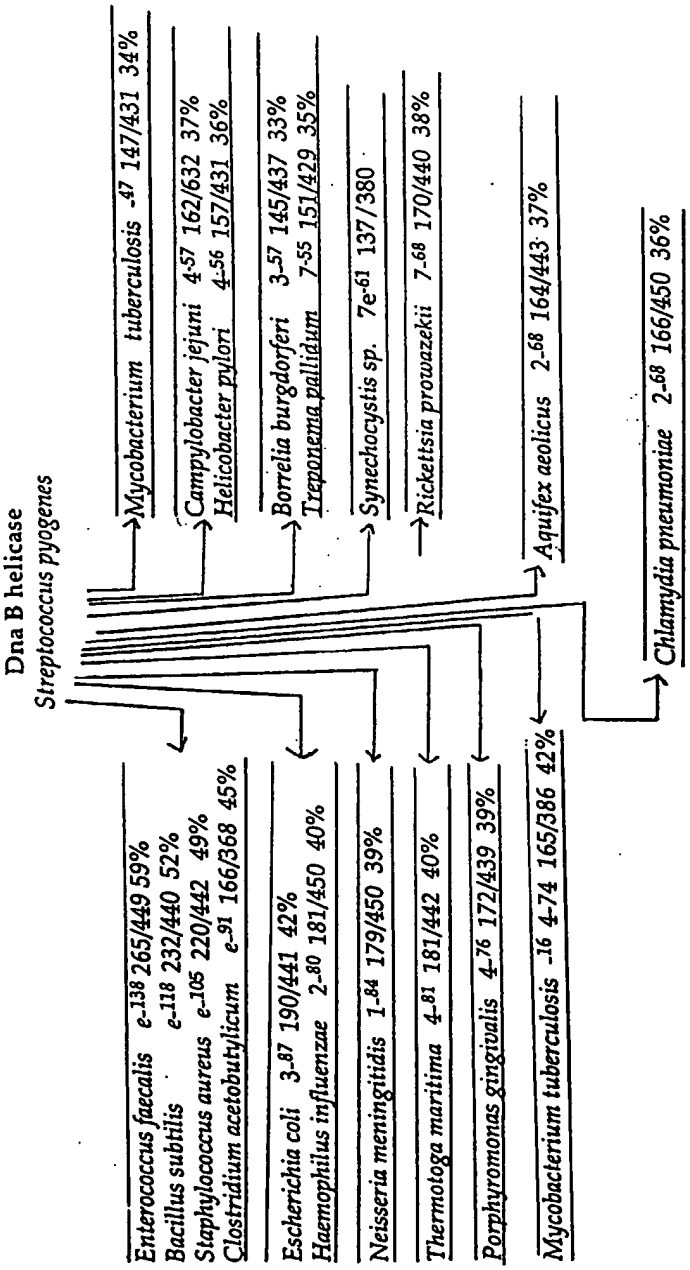
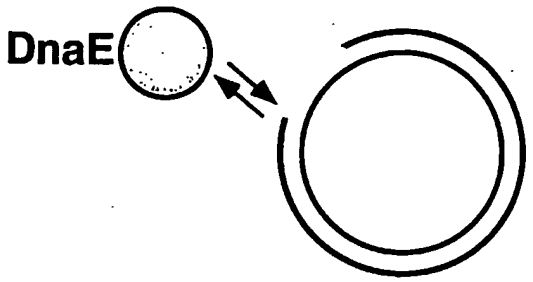
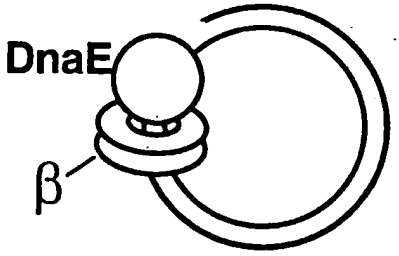

Dna B helicase
Streptococcus pyogenes
Mycobacterium tuberculosis e-47 147/431 34%
Campylobacter jejuni 4e-57 162/632 37%
Helicobacter pylori 4e-56 157/431 36%
Borrelia burgdorferi 3e-57 145/437 33%
Treponema pallidum 7e-55 151/429 35%
Synechocystis sp. 7e-61 137/380
Rickettsia prowazekii 7e-68 170/440 38%
Aquifex aeolicus 2e-68 164/443 37%
Chlamydia pneumoniae 2e-68 166/450 36%
Enterococcus faecalis e-138 265/449 59%
Bacillus subtilis e-118 232/440 52%
Staphylococcus aureus e-105 220/442 49%
Clostridium acetobutylicum e-91 166/368 45%
Escherichia coli 3e-87 190/441 42%
Haemophilus influenzae 2e-80 181/450 40%
Neisseria meningitidis 1e-84 179/450 39%
Thermotoga maritima 4e-81 181/442 40%
Porphyromonas gingivalis 4e-76 172/439 39%
Mycobacterium tuberculosis e-16 4e-74 165/386 42%

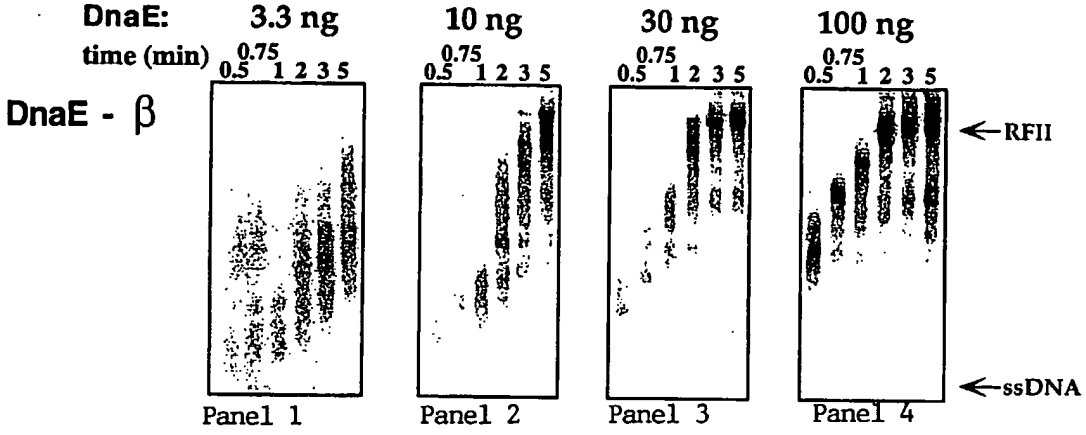
FIGURE 20H



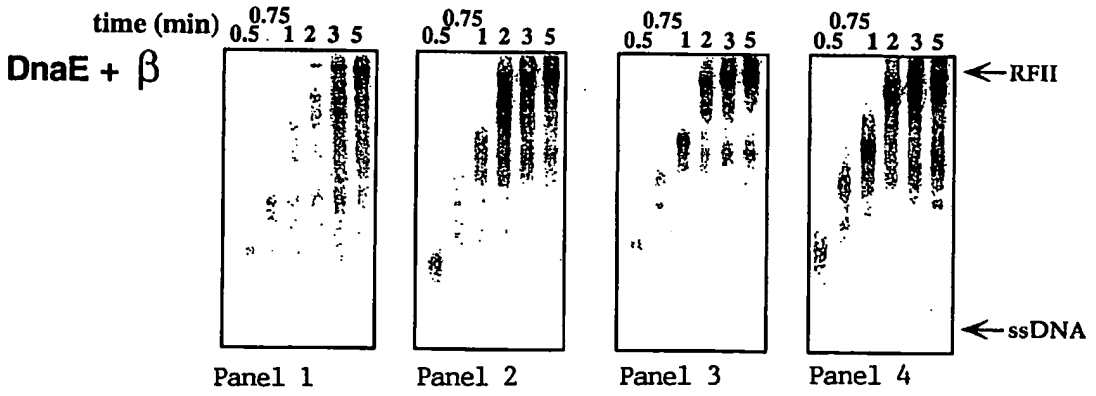
A



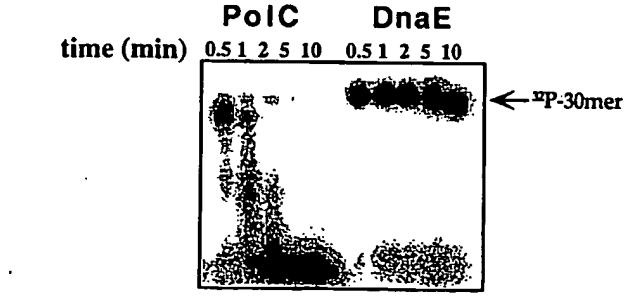
B



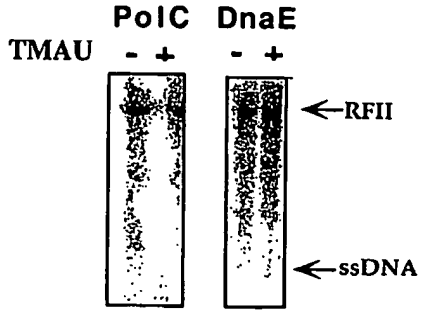
C



D



E



F

FIGURES 21A-F

SEQUENCE LISTING

<110> The Rockefeller University

<120> DNA REPLICATION PROTEINS OF GRAM POSITIVE BACTERIA AND THEIR USE TO SCREEN FOR CHEMICAL INHIBITORS

<130> 22221/1022

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<150> 60/146,178
<151> 1999-07-29

<160> 84

<170> PatentIn Ver. 2.1

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<213> Staphylococcus aureus

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Phe Tyr Asp Ala Cys Ile Ala Asn Asn Ile Lys Pro Ile Phe Gly Met
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Thr Ile Tyr Val Thr Asn Gly Leu Asn Thr Val Glu Thr Val Val Leu
 65                  70                  75                  80

Ala Lys Asn Asn Asp Gly Leu Lys Asp Leu Tyr Gln Leu Ser Ser Glu
                 85                  90                  95

Ile Lys Met Asn Ala Leu Glu His Val Ser Phe Glu Leu Leu Lys Arg
            100                 105                 110

Phe Ser Asn Asn Met Ile Ile Ile Phe Lys Lys Val Gly Asp Gln His
        115                 120                 125

Arg Asp Ile Val Gln Val Phe Glu Thr His Asn Asp Thr Tyr Met Asp
    130                 135                 140

His Leu Ser Ile Ser Ile Gln Gly Arg Lys His Val Trp Ile Gln Asn
145                 150                 155                 160

Val Cys Tyr Gln Thr Arg Gln Asp Ala Asp Thr Ile Ser Ala Leu Ala
                165                 170                 175

Ala Ile Arg Asp Asn Thr Lys Leu Asp Leu Ile His Asp Gln Glu Asp
            180                 185                 190

Phe Gly Ala His Phe Leu Thr Glu Lys Glu Ile Asn Gln Leu Asp Ile
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Asn Gln Glu Tyr Leu Thr Gln Val Asp Val Ile Ala Gln Lys Cys Asp
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Ala Glu Leu Lys Tyr His Gln Ser Leu Leu Pro Gln Tyr Glu Thr Pro
225                 230                 235                 240

Asn Asp Glu Ser Ala Lys Lys Tyr Leu Trp Arg Val Leu Val Thr Gln
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Leu Lys Lys Leu Glu Leu Asn Tyr Asp Val Tyr Leu Glu Arg Leu Lys
            260                 265                 270

Tyr Glu Tyr Lys Val Ile Thr Asn Met Gly Phe Glu Asp Tyr Phe Leu
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Ile Val Ser Asp Leu Ile His Tyr Ala Lys Thr Asn Asp Val Met Val
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Phe Leu Asn Pro Glu Arg Val Thr Met Pro Asp Ile Asp Ile Asp Phe
            340                 345                 350

Glu Asp Thr Arg Arg Glu Arg Val Ile Gln Tyr Val Gln Glu Lys Tyr
        355                 360                 365

Gly Glu Leu His Val Ser Gly Ile Val Thr Phe Gly His Leu Leu Ala
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Arg Ala Val Ala Arg Asp Val Gly Arg Ile Met Gly Phe Asp Glu Val
385                 390                 395                 400

Thr Leu Asn Glu Ile Ser Ser Leu Ile Pro His Lys Leu Gly Ile Thr
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Leu Asp Glu Ala Tyr Gln Ile Asp Asp Phe Lys Glu Phe Val His Arg
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Asn His Arg His Glu Arg Trp Phe Ser Ile Cys Lys Lys Leu Glu Gly
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Leu Pro Arg His Thr Ser Thr His Ala Ala Gly Ile Ile Ile Asn Asp
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His Pro Leu Tyr Glu Tyr Ala Pro Leu Thr Lys Gly Asp Thr Gly Leu
465                 470                 475                 480

Leu Thr Gln Trp Thr Met Thr Glu Ala Glu Arg Ile Gly Leu Leu Lys
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Ile Asp Phe Leu Gly Leu Arg Asn Leu Ser Ile Ile His Gln Ile Leu
            500                 505                 510

Thr Gln Val Lys Lys Asp Leu Gly Ile Asn Ile Asp Ile Glu Lys Ile
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Pro Phe Asp Asp Gln Lys Val Phe Glu Leu Leu Ser Gln Gly Asp Thr
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Thr Gly Ile Phe Gln Leu Glu Ser Asp Gly Val Arg Ser Val Leu Lys
545                 550                 555                 560
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Lys Leu Lys Pro Glu His Phe Glu Asp Ile Val Ala Val Thr Ser Leu
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Tyr Arg Pro Gly Pro Met Glu Glu Ile Pro Thr Tyr Ile Thr Arg Arg
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His Asp Pro Ser Lys Val Gln Tyr Leu His Pro His Leu Glu Pro Ile
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Gly Ala Phe Asp Ala Phe Gly Lys Thr Arg Ser Thr Leu Leu Gln Ala
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Ile Asp Gln Val Leu Asp Gly Asp Leu Asn Ile Glu Gln Asp Gly Ph
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Leu Phe Asp Ile Leu Thr Pro Lys Gln Met Tyr Glu Asp Lys Glu Glu
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Leu Pro Asp Ala Leu Ile Ser Gln Tyr Glu Lys Glu Tyr Leu Gly Phe
865                 870                 875                 880

Tyr Val Ser Gln His Pro Val Asp Lys Lys Phe Val Ala Lys Gln Tyr
                885                 890                 895

Leu Thr Ile Phe Lys Leu Ser Asn Ala Gln Asn Tyr Lys Pro Ile Leu
            900                 905                 910

Val Gln Phe Asp Lys Val Lys Gln Ile Arg Thr Lys Asn Gly Gln Asn
        915                 920                 925

Met Ala Phe Val Thr Leu Asn Asp Gly Ile Glu Thr Leu Asp Gly Val
    930                 935                 940

Ile Phe Pro Asn Gln Phe Lys Lys Tyr Glu Glu Leu Leu Ser His Asn
945                 950                 955                 960

Asp Leu Phe Ile Val Ser Gly Lys Phe Asp His Arg Lys Gln Gln Arg
                965                 970                 975

Gln Leu Ile Ile Asn Glu Ile Gln Thr Leu Ala Thr Phe Glu Glu Gln
            980                 985                 990

Lys Leu Ala Phe Ala Lys Gln Ile Ile Ile Arg Asn Lys Ser Gln Ile
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Asp Met Phe Glu Glu Met Ile Lys Ala Thr Lys Glu Asn Ala Asn Asp
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Val Val Leu Ser Phe Tyr Asp Glu Thr Ile Lys Gln Met Thr Thr Leu
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Gly Tyr Ile Asn Gln Lys Asp Ser Met Phe Asn Asn Phe Ile Gln Ser
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Phe Asn Pro Ser Asp Ile Arg Leu Ile
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<212> PRT
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